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NOTE ON THE CONVERGENCE OF APPROXIMATING RATIONAL FUNCTIONS OF PRESCRIBED TYPE

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Communicated September 3, 1963

By a *rational function of type* (j, k) we understand a function of the complex variable

$$r_{jk}(z) \equiv \frac{a_0 z^j + a_1 z^{j-1} + \ldots + a_j}{b_0 z^k + b_1 z^{k-1} + \ldots + b_k}, \quad \Sigma |b_i| \neq 0.$$

If the function $f(z)$ is continuous on a closed bounded set E, there exists under suitable conditions a rational function of type (j, k) of best approximation to $f(z)$ on E, namely, minimizing the Tchebycheff (uniform) norm

$$[\max \mid f(z) - r_{jk}(z) \mid, z \text{ on } E] \tag{1}$$

among all functions of type (j, k). These minimizing functions can be arranged in a table[1] which is analogous to that of Padé:

$$\begin{array}{l} r_{00}, r_{10}, r_{20}, \ldots \\ r_{01}, r_{11}, r_{21}, \ldots \\ r_{02}, r_{12}, \ldots \\ \ldots\ldots\ldots\ldots \end{array}$$

The first row consists merely of polynomials. It is appropriate to study the convergence of various sequences formed from this table. I have recently studied[2, 3] the convergence of certain rows of the table and now wish to indicate some results concerning the convergence of columns.

To be more specific, we have[3]

Theorem 1. *Let E be a closed bounded set whose complement is connected, and regular in the sense that it possesses a Green's function $G(z)$ with pole at infinity. Let E_σ denote generically the locus $G(z) = \log \sigma (> 0)$. Let the function $f(z)$ be analytic on E, meromorphic with precisely ν poles interior to E_ρ, $1 < \rho \leqq \infty$. Let the rational functions $r_{n\nu}(z)$ of respective types (n,ν) satisfy*

$$\limsup_{n \to \infty} \| f(z) - r_{n\nu}(z) \|^{1/n} \leqq 1/\rho, \tag{2}$$

where the norm is as in (1). Then for n sufficiently large the function $r_{n\nu}(z)$ has precisely ν finite poles, which approach respectively the ν poles of $f(z)$ interior to E_ρ. If

D denotes the interior of E_ρ with the ν poles of $f(z)$ deleted, the sequence $r_{n\nu}(z)$ converges to $f(z)$ throughout D, and for any closed bounded set S in D and in the closed interior of E_σ, $1 < \sigma < \rho$, we have

$$\limsup_{n\to\infty} [\max \mid f(z) - r_{n\nu}(z) \mid, z \text{ on } S]^{1/n} \leqq \sigma/\rho. \tag{3}$$

If the $r_{n\nu}(z)$ are the rational functions of type (n,ν) of best approximation to $f(z)$ on E in the sense of Tchebycheff, then (2) is satisfied.

Thus far, the $r_{n\nu}(z)$ need not be defined for every n, but below they shall be so defined.

Whether the $r_{n\nu}(z)$ are extremal or not, let ρ be the largest number such that $f(z)$ is meromorphic with precisely ν poles interior to E_ρ. If (2) holds, then (2) holds with the equality sign, as does (3) with $S = E_\sigma$, provided $1 < \sigma < \rho$, and provided no pole of $f(z)$ lies on E_σ.

As a complement to Theorem 1 we shall proceed to prove

THEOREM 2. *Let E and E_σ be as in Theorem 1. Let the function $F(z)$ be analytic and different from zero on E, meromorphic with precisely ν zeros interior to E_ρ, $1 < \rho \leqq \infty$. Let the rational functions $R_{\nu n}(z)$ of respective types (ν,n) satisfy for the Tchebycheff norm on E*

$$\limsup_{n\to\infty} \| F(z) - R_{\nu n}(z) \|^{1/n} \leqq 1/\rho. \tag{4}$$

Then the poles of the $R_{\nu n}(z)$ interior to E_ρ approach either E_ρ or the respective poles of $F(z)$ interior to E_ρ, multiplicities included. If D_1 denotes the interior of E_ρ with the poles of $F(z)$ deleted, the functions $R_{\nu n}(z)$ converge to $F(z)$ throughout D_1, and for any closed bounded set S in D_1 and in the closed interior of E_σ we have

$$\limsup_{n\to\infty} [\max \mid F(z) - R_{\nu n}(z) \mid. z \text{ on } S]^{1/n} \leqq \sigma/\rho. \tag{5}$$

If the $R_{\nu n}(z)$ are the rational functions of type (ν,n) of best approximation to $F(z)$ on E in the sense of Tchebycheff, then (4) is satisfied.

Thus far, the $R_{\nu n}(z)$ need not be defined for every n, but below they shall be so defined.

Whether the $R_{\nu n}(z)$ are extremal or not, let ρ be the largest number such that $F(z)$ is meromorphic with precisely ν zeros interior to E_ρ, $1 < \rho \leqq \infty$. If (4) holds, then (4) holds with the equality sign, as does (5) with $S = E_\sigma$, provided $1 < \sigma < \rho$, and provided no pole of $F(z)$ lies on E_σ.

Theorems 1 and 2 are essentially equivalent to each other with interchange of the roles of zeros and poles.

We proceed to prove Theorem 2. We write on E

$$\frac{1}{F(z)} - \frac{1}{R_{\nu n}(z)} \equiv \frac{R_{\nu n}(z) - F(z)}{F(z)R_{\nu n}(z)}. \tag{6}$$

For n sufficiently large, the denominator $F(z)R_{\nu n}(z)$ is bounded from zero, by (4). Then with the notation $f(z) = 1/F(z)$, $r_{n\nu}(z) = 1/R_{\nu n}(z)$ inequality (2) follows from (4) and (6), so Theorem 1 applies. If α is an arbitrary zero of $F(z)$ interior to E_ρ, let C be a circle in D_1 whose center is α, which contains on or within it no pole of $F(z)$ and no zero of $F(z)$ other than α. Then α is a pole of $f(z)$ interior to E_ρ, but $f(z)$ is analytic and different from zero on C, as is $r_{n\nu}(z)$ for n sufficiently large; $r_{n\nu}(z)$ converges to $f(z)$ uniformly on C. The functions $F(z)$ and $R_{\nu n}(z)$ are for n sufficiently large analytic on and within C; hence, by the equation

$$\frac{1}{f(z)} - \frac{1}{r_{n\nu}(z)} \equiv \frac{r_{n\nu}(z) - f(z)}{f(z)r_{n\nu}(z)} \tag{7}$$

$R_{\nu n}(z)$ converges uniformly on and within C to $F(z)$. For n sufficiently large the functions $R_{\nu n}(z)$ have precisely ν finite zeros, which approach, respectively, the ν zeros of $F(z)$ interior to E_ρ. The functions $R_{\nu n}(z)$ converge to $F(z)$ throughout D_1.

By Hurwitz's theorem, each zero of $f(z)$ interior to E_ρ is approached by an equal multiplicity of zeros of $r_{n\nu}(z)$, so each pole of $F(z)$ interior to E_ρ is approached by an equal multiplicity of poles of $R_{\nu n}(z)$. No point (not even a pole of $f(z)$) interior to E_ρ not a zero of $f(z)$ is approached by zeros of the respective $r_{n\nu}(z)$ (compare Lemma 1 of ref. 2), so no point interior to E_ρ not a pole of $F(z)$ is approached by poles of the respective $R_{\nu n}(z)$.

For such a point set as the circumference C just considered, inequality (3) applies, where S is C. By equation (7), inequality (5) likewise applies, where S is the closed interior of C. In order to prove (5) in Theorem 2, we choose τ, $\sigma < \tau < \rho$, so that no zeros or poles of $F(z)$ lie on E_τ. With center each zero of $F(z)$ on S, let a circle C (as before) be constructed so that the union S' of S and the closed interiors K of those circles lies interior to E_τ. Then by (3) for z on S–K and by (7), and by (5) for z on K with σ replaced by τ, we have

$$\limsup_{n\to\infty} [\max |F(z) - R_{\nu n}(z)|, z \text{ on } S']^{1/n} \leqq \tau/\rho,$$

an inequality valid also for z on S. If we replace S' by S and allow τ to approach σ, inequality (5) follows.

Again, by (7) and (2) we have for the Tchebycheff norm

$$\limsup_{n\to\infty} \left\| F(z) - \frac{1}{r_{n\nu}(z)} \right\|^{1/n} \leqq 1/\rho, \tag{8}$$

where the $r_{n\nu}(z)$ are now functions of types (n, ν) of best approximation to $f(z)$ on E. Since (8) holds for certain functions $1/r_{n\nu}(z)$ of types (ν, n), it holds also for the functions $R_{\nu n}(z)$ of best approximation to $F(z)$ on E; that is to say, (4) holds for the latter functions.

Under the conditions of the last paragraph of Theorem 2, suppose (4) holds with the inequality sign. Then by (6) it follows that (2) holds with the inequality sign, which contradicts Theorem 1. Under the same hypothesis, it follows by the method just used that (5) holds with the equality sign and with $S = E_\sigma$, provided no pole or zero of $F(z)$ lies on E_σ; the corresponding values of σ are everywhere dense in the interval $1 < \sigma < \rho$. If for a particular E_{σ_1}, interior to E_ρ and passing through a zero but no pole of $F(z)$, the first member of (5) with $S = E_{\sigma_1}$, is σ_0/ρ, less than σ_1/ρ, we choose a neighboring τ, $1 < \tau < \sigma_1$, so that no pole of $F(z)$ lies between E_{σ_1} and E_τ, and that no zero or pole lies on E_τ. Then for all E_σ, $\tau < \sigma < \sigma_1$, the first member of (5) with $S = E_\sigma$ is not greater than $[\max(\sigma_0, \tau)]/\rho$, which is less than σ_1/ρ; this contradicts (5) with $S = E_\sigma$ and with $[\max(\sigma_0, \tau)] < \sigma < \sigma_1$ as just proved when no pole or zero of $F(z)$ lies on E_σ. The proof of Theorem 2 is complete.

It is indicated[3] that with the hypothesis of the last paragraph of Theorem 1, and if $f(z) \equiv 0$ on no component of E, each point z_0 of E_ρ is a limit point of zeros of the $r_{n\nu}(z)$; consequently with the hypothesis of the last paragraph of Theorem 2, each

point z_0 of E_ρ is a limit point of poles of the $R_{\nu n}(z)$. This conclusion is true even if $\rho = \infty$, for then the only finite limit points of the poles of the $R_{\nu n}(z)$ are the poles of $F(z)$, where the multiplicity of the pole of $F(z)$ equals the multiplicity of the poles of the $R_{\nu n}(z)$ approaching it.

As has been indicated by the statements of Theorems 1 and 2, in the former there are few poles of the $r_{n\nu}(z)$ and their limit points are the poles of $f(z)$ interior to E_ρ; in the latter there are few zeros of the $R_{\nu n}(z)$ and their limit points are the zeros of $F(z)$ interior to E_ρ. If the poles α_j of $f(z)$ can be so ordered that $G(\alpha_1) < G(\alpha_2) < \ldots < G(\alpha_\mu)$, and if $f(z)$ has these but no other singularities interior to E_τ, then for each ν not greater than μ, the limit points of the poles of the $r_{n\nu}(z)$ are the first ν poles $\alpha_1, \alpha, \ldots, \alpha_\nu$. If the zeros α_j of $F(z)$ interior to E_τ can be similarly ordered, and if $F(z)$ is meromorphic interior to E_τ, then for each ν not greater than μ the limit points of the zeros of the $R_{\nu n}(z)$ are the first ν zeros $\alpha_1, \alpha_2, \ldots, \alpha_\nu$.

The present note deals primarily with the Tchebycheff norm, but under suitable circumstances the methods of reference 2 apply to a pth power norm and yield an analogue of Theorem 2.

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*VARIATIONAL BOUNDARY VALUE PROBLEMS FOR QUASI-LINEAR ELLIPTIC EQUATIONS, III**

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Communicated by A. Zygmund, July 29, 1963

In two preceding notes under the same title[1] (which we shall refer to below as (I) and (II), respectively), we have proved existence and uniqueness theorems for solutions of variational boundary value problems for quasi-linear elliptic differential equations of order $2m$ ($m \geq 1$) of the form

$$Au = \sum_{|\alpha| \leq m} D^\alpha A_\alpha(x, u, \ldots, D^m u) = f. \tag{1}$$

In the present note we shall give a method of establishing the existence of solutions of such problems under drastically weaker hypotheses than those of (I) and (II) but with the loss of a proof of uniqueness. The method which we shall apply is a variant of our preceding method, which was an orthogonal projection argument involving nonlinear operators in Hilbert space satisfying very weak continuity conditions. This variant applies in addition a form of the Leray-Schauder theory of the degree for completely continuous displacements in a Banach space.[2]

Section 1.—We apply the notation of our preceding notes.[1] On the open set Ω of R^n, we consider the quasi-linear differential operator A defined by (1) above,

where for each multi-index $\alpha = (\alpha_1, \ldots, \alpha_n)$, A_α is a function of x in Ω and of the function u on Ω and all its derivatives of order $\leq m$.

(We shall speak only of scalar functions u, but the argument and the conclusions obtained apply without any changes whatever to the case where u is an r-vector function and A represents a strongly elliptic system.)

Our first assumption upon the differential operator A is the same as Assumption 1 of (I), namely:

ASSUMPTION 1. *The functions A_α are continuous functions of all their arguments, x, u, and $p_\alpha = D^\alpha u$ for $|\alpha| \leq m$. There exists a continuous function $g(r)$ of the real variable r (with g otherwise unrestricted) such that for all u in $W^{m,2}(\Omega)$,*

$$\sum_{|\alpha| \leq m} |A_\alpha(x, u(x), \ldots, D^m u(x))|^2 \leq g(||u||_m) \sum_{|\beta| \leq m} |D^\beta u(x)|^2. \tag{2}$$

To define a variational boundary value problem for A, we assume a given closed subspace V of $W^{m,2}(\Omega)$ containing $C_c^\infty(\Omega)$. We state our generalized ellipticity assumption upon the boundary value problem defined by the pair (A, V) by using the generalized Dirichlet form $a(u, v)$ defined for u and v in $W^{m,2}(\Omega)$ by

$$a(u, v) = \sum_{|\alpha| \leq m} < A_\alpha(x, u, \ldots, D^m u), D^\alpha v > \tag{3}$$

where $<w, w_1>$ is the inner product in $L^2(\Omega)$. The form $a(u, v)$ is well defined because Assumption 1 implies that each $A_\alpha(x, u, \ldots, D^m u)$ lies in $L^2(\Omega)$ for each u in $W^{m,2}(\Omega)$.

Our second assumption will be stated in two forms, each considerably weaker than the assumptions of (II). The first of these is the following:

ASSUMPTION 2. *Ω is a bounded open set whose boundary is sufficiently smooth so that the imbedding map of $W^{m,2}(\Omega)$ into $W^{m-1,2}(\Omega)$ is compact. There exists a constant $k \geq 0$ such that for all u and v in V,*

$$Re\{a(u, u - v) - a(v, u - v)\} \geq -k||u - v||_{m-1}^2 .$$

A variant of Assumption 2 which is intuitively interesting and somewhat weaker may be stated as follows:

ASSUMPTION 2′. *Ω is a bounded open set whose boundary is sufficiently smooth so that the imbedding map of $W^{m,2}(\Omega)$ into $W^{m-1,2}(\Omega)$ is compact. There exists a linear differential operator B of order $\leq (2m - 1)$ of the form*

$$Bu = \sum_{|\alpha| \leq m, |\beta| \leq m-1} D^\alpha(b_{\alpha\beta}(x) D^\beta u)$$

with uniformly bounded coefficients $b_{\alpha\beta}$ on Ω, such that if $b(u, v)$ is the Dirichlet form corresponding to B, i.e.,

$$b(u, v) = \sum_{|\alpha| \leq m, |\beta| \leq m-1} < b_{\alpha\beta}(x) D^\beta u, D^\alpha v >,$$

then for all u and v in V,

$$Re\{a(u, u - v) - a(v, u - v) + b(u - v, u - v)\} \geq 0.$$

Obviously Assumption 2 is the special case of Assumption 2′ obtained by setting $Bu = \sum_{|\alpha| \leq m-1} D^\alpha(D^\alpha u)$.

DEFINITION. *For f in $L^2(\Omega)$ or in V^*, u in $W^{m,2}(\Omega)$ is said to be a weak solution of the equation $Au = f$ satisfying the null variational boundary conditions corresponding to the space V, if u lies in V and if for all v in V,*

$$a(u, v) = \langle f, v \rangle.$$

Our basic result in the present note is the following:

THEOREM 1. *Let A be a quasi-linear elliptic differential operator of the form (1) above, V a subspace of $W^{m,2}(\Omega)$ containing $C_c^\infty(\Omega)$. Suppose that A satisfies Assumption 1 and that the pair (A, V) satisfy Assumption 2′ (or the stronger Assumption 2). Suppose further that there exists a nonincreasing positive function $c(r)$ of the real variable r with $\lim_{r \to \infty} rc(r) = +\infty$, such that*

$$Re\{a(u, u)\} \geq c(\|u\|_m) \|u\|_m^2$$

for all u in V.

Then for each f in $L^2(\Omega)$ or V^*, there exists at least one weak solution u of $Au = f$ satisfying the null variational boundary conditions corresponding to V.

Following the pattern of our previous notes, we shall derive Theorem 1 in Section 3 from the results on nonlinear operators in Hilbert space derived in section 2 immediately below.

Section 2.—Let H be a Hilbert space with inner product (u,v), G a mapping of an open subset of H into H. G is said to be hemicontinuous if for each pair of elements u and w of H, the function $t \to G(u + tw)$ is continuous from the unit interval in t into the weak topology on H.

LEMMA 1. *Let G_0 be a hemicontinuous mapping of the open set D of H into H. Let u_0 and w_0 be a pair of elements of H such that u_0 lies in D and for all u in D,*

$$Re\ (G_0(u) - w_0, u - u_0) \geq 0. \tag{4}$$

Then $w_0 = G_0(u_0)$.

Lemma 1 is a generalization to hemicontinuous mappings G_0 of a theorem of G. Minty[3] for continuous monotone mappings.

Proof of Lemma 1: For $t > 0$, set $u_t = u_0 + t(w_0 - G_0(u_0))$, $v_t = G_0(u_t)$. Since D is open, u_t lies in D for $t < t_0$ for some $t_0 > 0$. Replacing u in (4) by u_t, we obtain after cancelling out $t > 0$,

$$Re\ (v_t - w_0, w_0 - G_0(u_0)) \geq 0,$$

or (after an obvious calculation),

$$Re\ (v_t - G_0(u_0), w_0 - G_0(u_0)) \geq \|w_0 - G_0(u_0)\|^2. \tag{5}$$

By hemicontinuity, v_t converges weakly to $G_0(u_0)$ as $t \to 0$. The left-hand side of (5) goes to 0 as $t \to 0$. The right-hand side of (5) is independent of t. Hence, $w_0 = G_0(u_0)$.

LEMMA 2. *Let G_0 be a hemicontinuous mapping of the open set D of H into H such that $Re\ (G_0(u) - G_0(v), u - v) \geq 0$ for all u, v in H. Let C be a compact (not necessarily linear) map of H into H, $G = G_0 + C$. Suppose that $\{u_k\}$ is a sequence of elements of D such that u_k converges weakly to u_0 in D as $k \to \infty$, $G(u_k)$ converges strongly to v_0 in H as $k \to \infty$. Then $v_0 = G(u_0)$.*

Proof of Lemma 2: Since $\{u_k\}$ is weakly convergent, it is bounded. Since C is compact, it follows that $C(u_k)$ is strongly convergent to $C(u_0)$. Hence, $G_0(u_k)$ converges strongly to $w_0 - C_0(u_0) = v_0$. For each u in D and each integer k,

$$Re\ (G_0(u) - G_0(u_k), u - u_k)) \geq 0.$$

Since the first term converges strongly to v_0 as $k \to \infty$ and the second term converges weakly to u_0, it follows for the limit that

$$Re\ (G_0(u) - v_0, u - u_0) \geq 0.$$

Since this is true for all u in D, it follows from Lemma 1 that $v_0 = G_0(u_0)$, i.e., $w_0 = G(u_0)$, and Lemma 2 is proved.

THEOREM 2. *Let G be a hemicontinuous mapping of H into H which maps bounded sets into bounded sets, C a compact nonnegative linear mapping of H into H. Suppose that for all u and v in H,*

$$Re\ (G(u) - G(v), u - v) + (C(u) - C(v), u - v) \geq 0.$$

Suppose further that there exists a continuous function $c(r)$ of the real variable r with $\lim_{r \to \infty} rc(r) = +\infty$ *such that*

$$Re\ (G(u), u) \geq c(||u||)\ ||u||^2. \tag{6}$$

Then G is onto.

Proof of Theorem 2: For each positive integer k, let $G_k = G + k^{-1} I$. (The device of introducing the G_k in the simpler case of continuous monotone operators has been used in an unpublished paper by George Minty on nonlinear Hammerstein integral equations.) Then if $T_k = G_k + C$, we see that T_k is hemicontinuous and for all u and v in H,

$$Re\ (T_k(u) - T_k(v), u - v) \geq k^{-1}||u - v||^2.$$

Applying Theorem 5 of (II), we know that for each k, T_k is one-to-one and onto and has a continuous inverse T_k^{-1} which maps bounded sets into bounded sets. We may write G_k in the form

$$G_k = T_k - C = (I - CT_k^{-1})T_k.$$

The range of G_k coincides with the range of $(I - CT_k^{-1})$ and since CT_k^{-1} is compact, the latter is a completely continuous displacement.

For each t with $0 \leq t \leq 1$, let $G_{k,t} = T_k - tC = (I - tCT_k^{-1})T_k$. For $t = 0$, $G_{k,0} = T_k$, while for $t = 1$, $G_{k,1} = G_k$. Let w be an arbitrary element of H. We shall show that w lies in the range of G_k by applying the theory of the Leray-Schauder degree.[2] For $t = 0$, w certainly lies in the range of T_k. Since $(I - tCT_k^{-1})$ is a continuous one-parameter family of completely continuous displacements of the Hilbert space H, it follows from the theory of the degree that in order to prove that w lies in the range of G_k (which equals the range of $(I - CT_k^{-1})$, it suffices to show that there exists a constant $N > 0$ such that if v is any solution of

$$(I - tCT_k^{-1})v = w, \tag{7}$$

for any t with $0 \leq t \leq 1$, then $||v|| \leq N$.

Suppose, however, that v is a solution of (6) and that $u = T_k^{-1}v$. Then

$$(I - tCT_k^{-1})T_k(u) = (T_k - tC)(u) = G_k u + (1 - t)Cu = w$$

or

$$G(u) + k^{-1}u + (1 - t)Cu = w.$$

Taking the real part of the inner product of the last equation with u, we have

$$Re\ (G(u),\ u) + k^{-1}\ ||u||^2 + (1 - t)(Cu,\ u) = (w,\ u),$$

and by the positivity of C, it follows that

$$Re\ (G(u),\ u) \leq ||w|| \cdot ||u|| \leq \left\{\frac{||w||}{||u||}\right\} ||u||^2.$$

By the hypothesis (6), it follows that $||u|| \leq N_1$ for a constant N_1 independent of t and indeed of k. Since $T_k = G + k^{-1} I + C$ maps bounded sets into bounded sets, $||v|| \leq N$. Hence, G_k is into H for each k.

We have, moreover, from the argument of the last paragraph a uniform constant N such that if $G_k u_k = w$, then $||u_k|| \leq N$. Choosing a weakly convergent subsequence of the sequence $\{u_k\}$, we have $G(u_k) = G_k(u_k) - k^{-1}u_k = w - k^{-1}u_k$ converging strongly to w. Hence, if u is the weak limit of the u_k, it follows from Lemma 2 that $G(u) = w$, and the proof of Theorem 2 is complete.

Remark: In the finite-dimensional case, a degree argument under slightly stronger hypotheses than (6) is an essential part of the discussion given by M. I. Vishik[4] in his treatment of weak solutions of quasi-linear elliptic equations. His method of passing from the finite-dimensional approximation to the general case is based upon another procedure utilizing strong a priori bounds.

Section 3.—We may now give a brief proof for Theorem 1.

Proof of Theorem 1: We proceed as in (II), translating the boundary-value problem into a nonlinear operator equation on the Hilbert space V using the operator G defined by $(G(u),\ v)_m = a(u,v)$, for all v in V. G is hemicontinuous under Assumption 1 by Lemma 3 of (II). G satisfies (6) by Assumption 2′ if we recall that $g(u,\ v) = (C_1 u, v)$, $v \in V$, defines a compact linear operator C_1 on V and if we choose C to be the positive part of $^1/_2(C_1 + C_1^*)$. The conclusion of Theorem 1 then follows from Theorem 2.

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*SOME EFFECTS OF SOLVATION UPON OPTICALLY ACTIVE MOLECULES**

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Solute-solvent interactions having chemically interesting consequences manifest themselves in many spectroscopic measurements. A study of such interactions requires the ability to perform dilute gas phase measurements that can serve as a reference standard for the optical properties of the "isolated" solute molecule. However, vapor phase measurements are not always feasible; hence, the concept of the "inert" solvent is widely employed. Briefly, one hopes to find a solvent in which, aside from unavoidable changes such as a variation in bulk dielectric constant, the molecular species of interest is in an environment that does not differ too markedly from that in the gas phase. For example, in spectroscopic studies of hydrogen bonding with alcohols, a hydrocarbon solvent such as hexane has been used[1] as such a reference medium.

In connection with our recent work[2, 3] on the temperature dependence of circular dichroism (C.D.), we have observed a number of solvent effects that have forced us to reappraise our estimations as to what might constitute an "inert" solvent. Our understanding of these effects is far from complete. Hence, our interpretative remarks can only be qualitative, but we feel that the observations themselves are of sufficient interest to merit mention at this time, especially since they also bear on recent optical rotatory dispersion (O.R.D.)[4] and C.D.[5] results for which no explanation could be offered.

The Composition of the Observed C.D. Curve.—The *observed* rotational strength $R_0{}^T$ associated with a particular absorption band at temperature T is conveniently gauged by the wavelength-weighted area under the corresponding C.D. curve according to the equation[6]

$$R_0{}^T \approx 0.696 \times 10^{-42} \int \frac{[\theta]}{\lambda} \, d\lambda \tag{1}$$

where $[\theta]$ is the molecular ellipticity. If, in fact, there are a number of molecular species present in equilibrium, all of which absorb in approximately the same wavelength region, the experimentally observed rotational strength $R_0{}^T$ is really a *population-weighted average* of the rotational contributions of the separate species, as are the experimental C.D. and O.R.D. curves. Any variation in conditions (e.g., temperature or solvent), which causes a change in relative population, will necessarily be reflected by a change in $R_0{}^T$ if the inherent rotational strengths of the separate species are sufficiently different. Such changes in population also imply changes in the observed spectral absorption band. However, because C.D. arises through a very slight difference in the ability of optically active molecules to absorb light of different circular polarizations, the intensity of the C.D. band is much more sensitive to a population variation than is the intensity of the corresponding absorption band. Moreover, because C.D. intensity is a signed quantity, transitions which frequently overlap to the point of indistinguishability in absorption

spectra are often at least partially discernible in the C.D. curve. As will be seen, this can often afford C.D. a distinct advantage over ordinary absorption for the study of solvent effects.

A Simple Case—Presence of a Single, Optically Active Species.—The simplest situation which could arise in solution is that of a single molecular species. In this case, $R_0{}^T$ is effectively independent of temperature. This is apparently true in cholestan-3-one (I) in EPA (ether-isopentane-alcohol) in the temperature interval[2] of $+25°$ to $-192°$C. In this range of temperatures, $R_0{}^T$ calculated[3] from equation (1) changes by less than 3%.

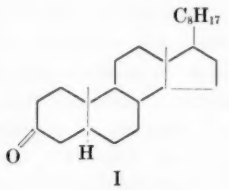
For experimental reasons, such low-temperature C.D. studies are restricted to a few solvents. However, if one extrapolates from the above results in EPA that there are no significant conformational alterations at room temperature in most common solvents, one can note the effect of solvation brought about by solvents of different character upon the same molecular framework (I). These data are collected in Table 1.

TABLE 1

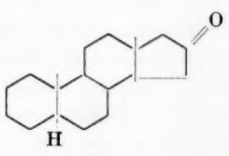
ROTATIONAL STRENGTH ($R_0{}^{298}$) OF CHOLESTAN-3-ONE (I)

Solvent	Rotational strength ($\times 10^{40}$ cgs)
$CHCl_3$	4.32
$(CH_3)CHOH$	3.95
CH_3OH	3.94
CH_2Cl_2	3.58
CH_3CN	3.45
CCl_4	3.41
$CH_3(CH_2)_3Cl$	3.32
$\overline{OCH_2CH_2OCH_2CH_2}$	3.17
C_6H_{12}	2.73
$(CH_3)_2CH(CH_2)_4CH_3$	2.52

It will be noted that the differences are of the order of one unit of rotational strength, as they are, in fact, in 5α-androstan-16-one (II) ($R_0{}^{298} = -15.7 \times 10^{-40}$ in cyclohexane and -17.8×10^{-40} in chloroform), for which lack of conformational mobility in EPA has also been demonstrated.[2] However, because II shows the typical,[7] strong Cotton effect of such fused cyclopentanones, while cholestan-3-one (I) exhibits a comparatively weak one, the effect of solvent change is apparently drastic for I and of minor significance for II on a percentage scale. On an absolute basis, the differences are actually very similar in magnitude.



I



II

Conformational Equilibria.—The effects caused by temperature on a conformational equilibrium are clearly distinguishable in Figure 1, where the equilibrium[8] affected is between the diequatorial (IIIa) and diaxial (IIIb) forms of *trans*-2-chloro-5-methylcyclohexanone. The data (EPA solvent) can be analyzed[3] quanti-

tatively on this assumption to yield a value of ΔF^0 and a relative conformer population in accord with that obtainable[9] by other methods. These data are also consistent with the supposition that $\Delta H^0 = \Delta F^0$, and $\Delta S^0 = 0$, as is the case for a simple *two*-conformer interconversion.

The absorption band (Fig. 1) observed in the vicinity of 300 mμ is a superposition of the separate bands due to the individual conformers which absorb at different wavelengths (*ca.* 20 mμ[10] apart). Nevertheless, the overlapping is so great that the separate contributions are hardly recognizable. However, the presence of two species is clearly evident in the C.D. curve, since the separate contributions, although severely overlapping, are of different sign. A similar conformational conclusion had been reached earlier[5] by O.R.D. measurements.

We emphasize here that, although a simple conformational equilibrium is presupposed, we do not assume the absence of solvation, but merely that the extent of such solvation is effectively constant over the temperature range covered.

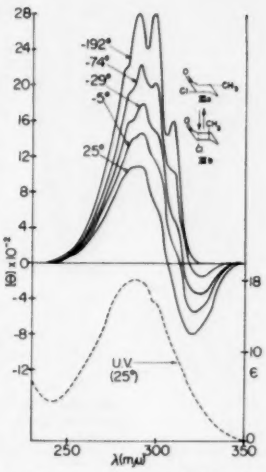


FIG. 1.—Circular dichroism curves (EPA) at 25°, −5°, −29°, −74°, and −192°C, as well as the ultraviolet absorption spectrum (EPA) at 25°C of (+)-*trans*-2-chloro-5-methylcyclohexanone (III).

Equilibria Associated with Solvation.—Like conformational equilibria, solvation equilibria can lead to a variety of species in solution. For example, the rigid bicyclic compound isofenchone (IV), kindly provided by Dr. A. Rassat,[4] can undergo no radical conformational change. Yet examination of its C.D. curve (Fig. 2) in methanol-ethanol (1:4) at room temperature clearly shows the presence of *at least two* different species with rotational strengths of opposite sign. The different bands could be indicative of dimerization, but in view of the high hydrogen-bonding power of the solvent, it seems more reasonable to postulate the simultaneous presence of solvated and unsolvated molecules which absorb at slightly different wavelengths and which have rotational strengths of opposite sign. Further evidence for solvation can be derived from temperature studies. As the temperature is lowered from 25 to −74°C, the short-wavelength (solvated) band grows at the expense of the long-wavelength (unsolvated) band (see Fig. 3).

Camphor (V), although rigid, also shows a variation (see Fig. 3) in $R_0{}^T$ with temperature. Again, a solvation equilibrium seems the most reasonable explanation. For such a solvation process, it is expected that ΔF^0 itself is a function of temperature, as can be reasonably deduced[3] from the experimental C.D. curves (Fig. 3).

It should be pointed out that postulation of a solvation equilibrium is not original with the present authors. Pariaud[11] was led to precisely the same conclusion from studies of variation in the specific rotation of camphor (V) at a few wavelengths with changes in temperature, concentration, and solvent. Moreover, he determined the melting point curves of camphor in cyclohexane, carbon tetrachloride, and benzene, all of which indicated the formation of weakly bound addition compounds between solute and solvent. That cyclohexane is not an "inert" solvent is perhaps

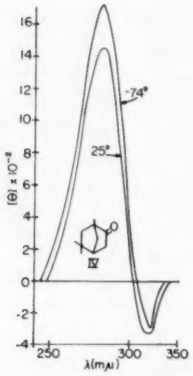


FIG. 2.—Circular dichroism curves (methanol-ethanol, 1:4) of isofenchone (IV) at 25° and −74°C.

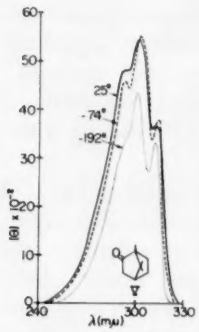


FIG. 3.—Circular dichroism curves (isopentane - methylcyclohexane, 5:1) of (+)-camphor (V) at 25°, −74°, and −192°C.

surprising, but nevertheless is in accord with the C.D. data.[12] In this connection, it is pertinent to note that cholestan-3-one (I) in isopentane-methylcyclohexane (5:1) exhibits not only a temperature-dependent $R_0{}^T$, but also a blue shift and loss of fine structure at lower temperatures (see Fig. 4). These features are indicative of increase in solvation compared to that existing at room temperature.

In the light of the above remarks, the drastic changes observed by Gervais and Rassat[4] in the O.R.D. curves of isofenchone (IV) and epiisofenchone in the 300 mμ region with changes in solvent become understandable. What these workers were seeing was the relative waxing and waning of the contributions of the solvated and unsolvated species to the observable optical activity as manifested in rotatory dispersion.

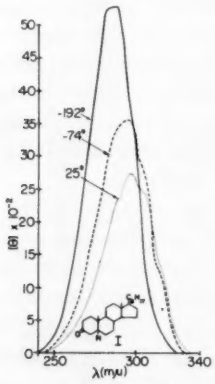


FIG. 4.—Circular dichroism curves (isopentane - methylcyclohexane, 5:1) of cholestan-3-one (I) at 25°, −74° and −192°C.

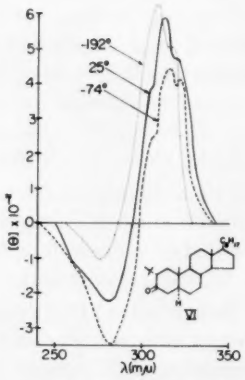


FIG. 5.—Circular dichroism curves (isopentane-methylcyclohexane, 6:1) of 2α-*t*-butylcholestan - 3 - one (VI) at 25°, −74°, and −192°C.

More Complicated Situations.—Clearly, combinations of conformational and solvational equilibria are possible, and such situations will show even more complex temperature variations. For example, in 2α-*t*-butylcholestan-3-one (VI)[13] in methylcyclohexane-isopentane (1:6), $R_0{}^T$ goes through a maximum as the temperature is lowered (Fig. 5), which can only be the case if there exist *more than two* forms in equilibrium. No halogen is present in the molecule, which might lead to a marked wavelength shift upon conformational interchange (cf. 2-chloro-5-methylcyclohexanone (III)), so that the fairly well-separated bands of opposite sign may reasonably be taken as evidence for the presence of solvated and unsolvated species. Moreover, various conformers are possible, so that the observed temperature dependence is most probably the result of both conformational and solvational equilibria.

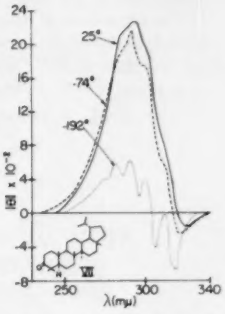


FIG. 6.—Circular dichroism curves (EPA) of lupan-3-one (VII) at 25°, −74°, and −192°C.

Another case not simply interpretable[5] is that of lupan-3-one (VII). The C.D. data (Fig. 6) in EPA are virtually independent of temperature from 25 to −41° and hence are indicative of a fairly static population distribution in this temperature range. This is in accord with what has been observed[14] by nuclear magnetic resonance measurements, albeit for a different solvent. However, below −41° there is a marked decrease in the magnitude of the C.D. curve in the short wavelength region. The presence of the relatively widely separated C.D. extrema of opposite sign is again indicative of at least one solvation equilibrium, although the C.D. behavior below −41° shows the situation to be complicated by still further equilibria.

Summary.—Temperature-dependent circular dichroism (C.D.) data are capable of providing information as to the nature and extent of both conformational and solvational equilibria. Moreover, in the latter case they indicate solute-solvent interactions in media such as hydrocarbons which are ordinarily considered unlikely to participate in compound formation. Such conclusions are much less easily reached on the basis of absorption spectroscopy, and the C.D. data point up the need for exercising care in deciding what might constitute an "inert" solvent.

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* Part XCIV in the Stanford series, *Optical Rotatory Dispersion Studies.* For preceding article, see ref. 3.

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[2] Wellman, K. M., E. Bunnenberg, and C. Djerassi, *J. Am. Chem. Soc.*, **85**, 1870 (1963).
[3] Moscowitz, A., K. M. Wellman, and C. Djerassi, *J. Am. Chem. Soc.*, in press.
[4] Gervais, H. P., and A. Rassat, *Bull. soc. chim. France*, 743 (1961).
[5] Witz, P., H. Herrmann, J.-M. Lehn, and G. Ourisson, *Bull. soc. chim. France*, 1101 (1963).
[6] Djerassi, C., *Optical Rotatory Dispersion*, (New York: McGraw-Hill, 1960), p. 164.
[7] Djerassi, C., W. Closson, and A. E. Lippman, *J. Am. Chem. Soc.*, **78**, 3163 (1956).
[8] Djerassi, C., L. E. Geller, and E. J. Eisenbraun, *J. Org. Chem.*, **25**, 1 (1960).
[9] Allinger, N. L., J. Allinger, L. E. Geller, and C. Djerassi, *J. Org. Chem.*, **26**, 6 (1960).
[10] See Cookson, R. C., *J. Chem. Soc.*, 282 (1954).

[11] Pariaud, J. C., *Bull. soc. chim. France*, 103 (1950).

[12] The formation of solute-solvent associations in nonpolar solvents has also been inferred from I.R. (see Bellamy, L. J., and R. L. Williams, *Trans. Faraday Soc.*, **55**, 14 (1959)) and N.M.R. data (see Hatton, J. V., and R. E. Richards, *Mol. Phys.*, **5**, 153 (1962)).

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NOTE ON THE LINEARIZED BOLTZMANN INTEGRAL EQUATION FOR RIGID SPHERE MOLECULES

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1. *Introduction.*—The linearized Boltzmann equation for a gas of hard spheres can be written in the spatially homogeneous case as:[1]

$$\frac{\partial f(\mathbf{v},t)}{\partial t} = -k(v)f(\mathbf{v},t) + \int d\mathbf{v}_1 G(\mathbf{v},\mathbf{v}_1)f(\mathbf{v}_1,t) \tag{1}$$

where

$$k(v) = 2\pi^2\left\{e^{-v^2} + \left(2v + \frac{1}{v}\right)\int_0^v dx e^{-x^2}\right\}$$

$$G(\mathbf{v},\mathbf{v}_1) = 2\pi\left\{(2/\rho)e^{-A/4} - \rho e^{-1/2(v^2+v_1^2)}\right\}$$

$$\rho = |v - v_1|;\ A = \rho^2 + (v^2 - v_1^2)^2/\rho^2.$$

The velocity $\mathbf{v}$ is measured in units $(kT/m)^{1/2}$ and the diameter of the sphere is taken as unit of length.

For the discussion of the initial value problem for equation (1), it is clearly of interest to consider the eigenvalue problem:

$$\lambda\psi(\mathbf{v}) = -k(v)\psi(\mathbf{v}) + \int d\mathbf{v}_1 G(\mathbf{v},\mathbf{v}_1)\psi(\mathbf{v}_1) \tag{2}$$

since the eigenvalues λ_i are directly related to the different times of relaxation to the equilibrium Maxwell distribution. Unfortunately, not much is known about the eigenvalue spectrum, beside the obvious fact that $\lambda = 0$ is a fivefold eigenvalue corresponding to the eigenfunctions 1, $\mathbf{v}$, and v^2. Recently, Pekeris[2] has shown explicitly that the kernel $K(\mathbf{v},\mathbf{v}_1)$ occurring in an associated eigenvalue problem,

$$\mu\phi(\mathbf{v}) = \int K(\mathbf{v},\mathbf{v}_1)\phi(\mathbf{v}_1)d\mathbf{v}_1, \tag{3}$$

where

$$K(\mathbf{v},\mathbf{v}_1) = \frac{G(\mathbf{v},\mathbf{v}_1)}{[k(v)k(v_1)]^{1/2}}$$

is *not* square-integrable. However, Carleman[3] has shown that the second iterate of

$K(\mathbf{v}, \mathbf{v}_1)$ is square-integrable, so that (3) has a discrete spectrum and a corresponding complete set of square-integrable eigenfunctions.[4]

2. The purpose of this note is to prove the following analogous result for the kernel $G(\mathbf{v}, \mathbf{v}_1)$:

THEOREM. *The third iterate of* $G(\mathbf{v}, \mathbf{v}_1)$ *is square-integrable.*

Proof: We start from the inequality due to Carleman:[5]

$$\int d\mathbf{v}_1 \frac{|G(\mathbf{v},\mathbf{v}_1)|^\beta}{(1+v_1)^\alpha} < \frac{C}{(1+v)^{\alpha+1}} \tag{4}$$

which holds for $1 \leq \beta \leq 3$ and for all $\alpha \geq 0$; C is a constant independent of α and β. Using (4) and Schwartz' inequality, it follows that the second iterate

$$G_2(\mathbf{v}, \mathbf{v}_1) = \int G(v_1,v_2)G(v_2, v_1)d\mathbf{v}_2$$

satisfies the inequality:

$$|G_2(v, v_1)| \leq \left[\int |G(v_1,v_2)|^2 dv_2\right]^{1/2} \cdot \left[\int |G(v_2,v_1)|^2 dv_2\right]^{1/2} < \frac{C_2}{(1+v)^{1/2}(1+v_1)^{1/2}},$$

This gives for the third iterate:[6]

$$|G_3(v, v_1)| \leq \int |G_2(v, v_2)| \cdot |G(v_2, v_1)|dv_2 < \frac{C_2}{(1+v)^{1/2}} \cdot \int d\mathbf{v}_2 \frac{|G(v_2,v_1)|}{(1+v_2)^{1/2}} < \frac{C_3}{(1+v)^{1/2}(1+v_1)^{3/2}},$$

and so on, leading to

$$|G_6(v, v_1)| < \frac{C_6}{(1+v)^{1/2}(1+v_1)^{5/2}}.$$

Consequently, since

$$G_6(v, v_1) = \int dv_2 G_3(v, v_2)G_3(v_2, v_1),$$

one can conclude:

$$\int dv_1 G_3^2(v, v_1) = G_6(v, v) < \frac{C_6}{(1+v)^5},$$

so that

$$\iint dv dv_1 G_3^2(v, v_1) < C_6 \frac{\pi}{3} < \infty. \tag{5}$$

3. One can conclude that the integral equation

$$\mu'\phi'(\mathbf{v}) = \int d\mathbf{v}_1 G(v, v_1)\phi'(v_1) \tag{6}$$

has a discrete spectrum and a complete set of square-integrable eigenfunctions,[4] but unfortunately this does not allow a complete discussion of the original eigenvalue problem (2). All one can say is that, beside the zero eigenvalue, the spectrum of λ will be continuous in the interval $(-\infty, -4\pi^2)$. This follows from the fact that $k(v)$ is monotonically increasing from $k(0) = 4\pi^2$ by using a theorem of H. Weyl and Von Neumann.[7] Physically this means that there are arbitrarily short relaxa-

tion times, which is not surprising since the relaxation times must for large velocities behave as $1/vn\sigma$, and the cross section σ is constant for hard spheres. It seems likely that in the interval $(-4\pi^2, 0)$ there are at least some discrete eigenvalues related to the viscosity and heat conduction of the gas, but no proof is available.[8]

[1] First shown by Hilbert in *Integral Gleichungen* (New York: Chelsea Publishing Co., 1953), chap. 22, p. 276. We will follow the presentation of Carleman, T., in *Problèmes Mathématiques dans la Théorie Cinétique de Gaz* (Uppsala: Almquist and Wilsells, 1957).

[2] Pekeris, C. L., these PROCEEDINGS, **49**, 38 (1963).

[3] Carleman, T., *op. cit.*, p. 77.

[4] For the appropriate theorems concerning completely continuous operators, see, for example, Schatten, R., in *Norm Ideals of Completely Continuous Operators* (Berlin: Springer Verlag, 1960), pp. 16–18.

[5] Carleman, T., *op. cit.*, p. 74.

[6] Although the estimate given here does not preserve the symmetry of $G_3(v, v_1)$, it is easy to show by the same methods that

$$|G_3(v, v_1)| < C\left(\frac{1}{(1+v)^{1/2}(1+v_1)^{3/2}} + \frac{1}{(1+v)^{3/2}(1+v_1)^{1/2}}\right),$$

which is symmetric, and so on for the higher iterates.

[7] Cf. Riesz, F., and Sz.-Nagy, B., in *Functional Analysis* (New York: F. Ungar Publishing Co., 1955), p. 367. The theorem as used here is as follows "THEOREM. If a completely continuous symmetric transformation B is added to a symmetric transformation A, the set of limit points of the spectrum remains invariant." We are indebted to Professor M. Schreiber for pointing this theorem out to us.

[8] *Note added in proof:* Similar results have been obtained by a different method by Grad, H., in *Third International Rarified Gas Symposium* (New York: Academic Press, 1963), vol. I, pp. 26–58.

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*AUDITORY RESPONSES IN THE TOKAY GECKO**

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Most lizards do not produce any vocal sounds, and the faint hiss occasionally heard from a number of them is perhaps only the result of a sudden expiration of air. A notable exception to this rule is the family Gekkonidae, whose members utter cries usually described as chirps or squeaks. Indeed, the name "Gecko" is supposed to be an imitation of the call of the animal.

All geckos are nocturnal, and it has been suggested that the development of vocal capabilities is in some sense a compensation for the lack of visual cues, especially in the mating process. Nearly all species in this family lack eyelids and are provided with an iris that closes almost completely under ordinary daytime illumination.

The species investigated here is the Tokay Gecko, and its scientific name is *Gekko gecko*. One feature is the presence on the dorsal surface of numerous randomly distributed scales that are larger than the others and usually bear a small knob or crest, giving a rough texture to the skin. These animals are distributed

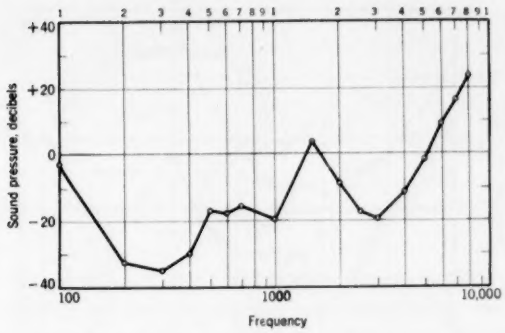

FIG. 1.—Sensitivity function for a lizard, *Gekko gecko*. The curve shows the sound pressure in decibels relative to 1 dyne per sq cm necessary to produce a cochlear potential of 0.1 μv at the frequencies indicated.

throughout Southeast Asia and are one of the commonest species there. They are now found also in a few regions of the United States, having stowed away in ship cargo and then escaped to establish themselves in some of the seaports of the South. Some of our specimens came from India, and others were collected in this country. This is a rather large species of lizard; our specimens measured from 20–23 cm over all, 11–13 cm from snout to vent, and weighed 42–73 gm.

Their attitude is belligerent; when disturbed they open the mouth to its fullest extent, utter a fairly loud cry that may be compared to the sound of a squeaky hinge without high-frequency components, and often follow by making quick lunges at an intruder. Also, if the opportunity is offered, they seize a finger and deliver a vigorous pinch.

The structure of the lizard ear has been studied by a number of anatomists, though not in the detail that is desirable, and many features remain obscure. Evidently there are large species variations in some portions of the mechanism.

In the gecko there is an external auditory meatus of oval cross section, measuring about 2 by 4 mm and obliquely placed at the side of the head. The orifice is provided with a constrictor muscle, and we have observed its closure when the

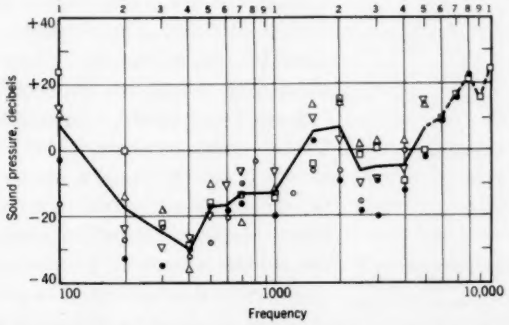

FIG. 2.—Sensitivity data for five geckos, as in Fig. 1. The curve represents mean values.

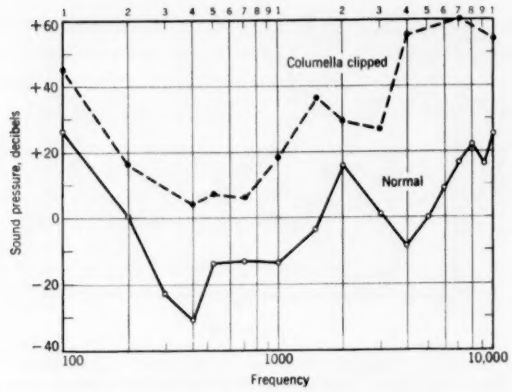

FIG. 3.—The sensitivity of a gecko ear under normal conditions and after interrupting the columella.

animal is disturbed. The opening leads inward about 3 mm to a tympanic membrane 4 by 7 mm in size. Extending from this membrane to the otic capsule is a columellar system made up of two portions. An extracolumella is attached to the tympanic membrane and continues medially to a thin rod, the columella proper, which expands at its innermost end to form the stapes lying in the oval window. There is a round window opening into the pharyngeal cavity, a space only slightly separated from the mouth.

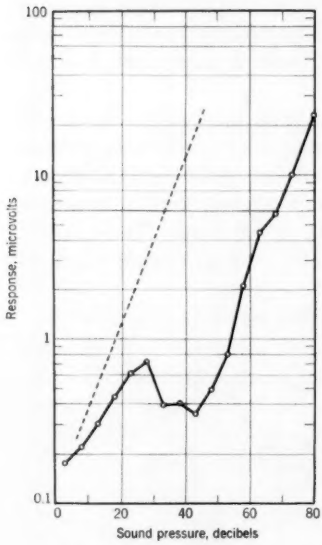

FIG. 4.—Variations of response as a function of sound pressure (solid line). The sound pressure is in decibels above 1 dyne per sq cm. The dotted line shows the slope of a linear function.

Procedure.—Our study of auditory sensitivity in this animal was carried out by placing an active electrode on the round window membrane, with an indifferent electrode in other tissue nearby, and observing the potentials produced on stimulating the ear with tones. The animals were anesthetized with 5% urethane (ethyl carbamate) in Ringer's solution. The dose level was 0.04 cc per gm of body weight. The round window was approached on the right side by an opening made through the lower jaw close to the posterior end of the mandible. The electrode was a silver bead about 0.1 mm in diameter, made by briefly heating a wire of 0.05 mm diameter in a flame.

Sounds were produced with a Western Electric 555 loudspeaker and conducted to the ear through a tube that ended in a sound cannula sealed tightly over the external meatus. A probe tube attached to a condenser microphone ran through the sound cannula and ended near its tip, and this system was calibrated to give measurements of sound pressure for the stimulating tones.

The body temperature of the animals was

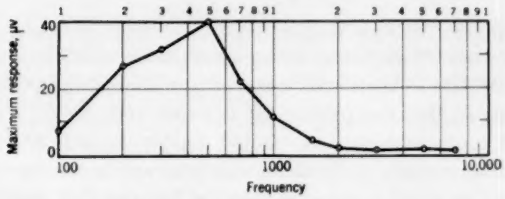

FIG. 5.—Maximum responses obtained for tones over the range 100–7000 cycles.

measured during the experiment and was found to remain in the region of 24°C.

Results.—The sensitivity was determined by presenting various tones at the sound pressure necessary to produce a response of 0.1 μv. Results for one animal are given in Figure 1. As indicated, responses were obtained over a range from 100–7000 cycles per sec, with the greatest sensitivity in the low tones from 200–400 cycles, and rapidly decreasing sensitivity for tones above 3,000 cycles.

Similar results for five animals are given in Figure 2. Here the different kinds of points represent the individual ears, and the curve shows the mean values. The individual variations are of the order of 20–30 db, but the forms of the functions are much the same for the different ears.

The role played by the columellar system in the transmission of sounds to the inner ear is reflected in the results of Figure 3. The solid line shows a normal sensitivity curve, with the columellar system intact, whereas the dashed curve shows the sensitivity after an interruption of the system made by clipping the columella in two places, leaving a gap. The losses as a result of this interruption range from 16 to 62 db, with a mean value of 31 db. In the cat a similar interruption of the ossicular chain has been found to impair the ear's sensitivity by an average amount of 28 db.

In numerous animals the cochlear potentials have been found to increase linearly as a function of sound pressure at low and intermediate levels, and then for extreme pressures to depart from linearity, pass through a maximum, and then decline sharply as overloading becomes severe. An attempt was made to obtain intensity functions of this kind for the gecko, but with little success. We found that when the sound pressure was raised even moderately above the level necessary to produce potentials of 0.1 μv the responses became highly irregular and often varied as much as 30 db during a single observation. We attribute these variations to the action of middle ear muscles.

An attempt to exclude the action of these muscles by increasing the depth of anesthesia—a procedure that works fairly well in mammals—was unsuccessful within the limit at which respiration was impaired and the animal died. We had the impression that the variability was reduced at deeper levels of anesthesia, but not to the point where reliable measurements were possible. Also, we were unable to fatigue the muscle action by the protracted application of strong tones; rather, this procedure seemed to produce a sensitization of the action so that even the fainter tones for a time gave variable responses.

Some results that illustrate the effects of the muscle action are given in Figure 4. Here the sound pressure was varied over a range of nearly 80 db for a tone of 500

cycles. The response rose at first at a rate a little below linearity, then fell sharply over a range of nearly 20 db before rising again, after which it continued to rise at practically a linear rate as the sound pressure was increased further. We interpret these results to mean that the middle ear muscles underwent in the beginning a slight increase of tonus and then contracted sharply around 30 db so as to reduce the transmission appreciably, increasing this contraction for the next two steps of stimulation. At this point it appears that the muscles had reached their limit of contraction, where they remained as the stimulation was further increased. The response therefore follows a linear course for this upper portion of the function, though it is about 35 db below what it would be without the muscle action. The dotted line on this graph suggests what the course of the response would be if the response continued to be linear beyond its lowermost values up to a sound pressure of 50 db (316 dynes per sq cm). We should not expect the linear course of the function to extend much farther than this because of the entrance of overloading at high levels.

We have referred here to "middle ear muscles," but we are not able at this time to be as specific as we would like about their number or identity. The anatomical literature on the gecko and on lizards in general gives scanty information on the aural muscles. There is evidence that a muscle is present in the gecko that is variously called a stapedius muscle (Killian[1]), or musculus laxator tympani (Gaupp[2]), but perhaps better named the musculus extracolumellaris (Versluys[3]). It runs from the hyoid arch to the posterior accessory process of the extracolumella. The extracolumellar structure is highly complex, and it is possible that other muscles are concerned in the restraint of the columellar motions. This question is being studied further.

Because of the irregularities in the auditory potentials at high levels we were usually not able to determine maximum responses to the different tones with any great degree of reliability. Figure 5 shows the results of our attempts at such measurements in one animal. Plotted here are the largest potentials that we were able to record under the conditions. The values shown for frequencies above 500 cycles we regard as fairly close to true maxima, but those for lower tones are probably reduced appreciably by muscle action and might well approach 40 μv if such action were excluded.

Discussion—The sensitivity shown by this ear is somewhat below that found by the cochlear potential method in mammals[4] when the comparison is made between the most favorable regions of frequency for each species. The cat, for example, shows the greatest sensitivity in the region of 7,000 cycles, and is superior to the gecko's sensitivity at 300 cycles by about 30 db. If we make the comparison in the low frequencies, the difference is less, though still in favor of the cat's ear. In comparison with other reptiles the gecko shows up a little more favorably. Its sensitivity lies about midway between that of snakes[5] and turtles;[6] it is about 10 db better than snakes and 10 db poorer than most turtles. However, it is inferior to the caiman[7] by about 30 db. Thus, we find among the reptiles a considerable range of sensitivity, with the lizards occupying an intermediate position. No doubt these variations will be found to correspond to differences in structure of the middle and inner ears, but these points remain for further exploration.

* This investigation was supported by grants from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service, aided by a contract with the Office of Naval Research, and by Higgins funds allotted to Princeton University. Permission is granted for reproduction and use by the United States Government.

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*COMPOSITION OF THE NUCLEUS AND CHROMOSOMES IN THE LAMPBRUSH STAGE OF THE NEWT OÖCYTE**

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A lampbrush chromosome is metabolically active at many sites all along its axis.[1, 2] This is in marked contrast to other interphase chromosomes, in which a few loci are active while most are relatively inactive,[3, 4] and also in striking contrast to mitotic chromosomes, which appear to be metabolically inert, though they are being moved about.[5] Since lampbrush chromosomes are so active metabolically, it is of interest to know about their composition and to compare it with the composition of other chromosomes. In this paper we present determinations of the deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein contents of lampbrush chromosomes and of the nuclei containing these chromosomes.

Lampbrush chromosomes occur in the growing oöcytes of many different animals.[6] In our work we have used oöcytes of the newt, *Triturus viridescens*. Chromosomes of ordinary size are present in the oöcyte both before and after the period of growth; presence of giant lampbrush chromosomes in the oöcyte only at the time of growth points, therefore, to a correlation between the massive synthesis of cytoplasm and the formation of the enormous chromosomes with their lateral extensions, the so-called loops.[7, 8]

Other well-known giant chromosomes are the polytene chromosomes of dipteran larvae.[3] The puffs of these chromosomes are sites of marked accumulation of protein and nucleic acid,[9, 10] and puffs of polytene chromosomes may perhaps be compared with the loops of lampbrush chromosomes, for both puffs and loops are

sites of activity. A difference between these structures is that although loops occur at almost all chromomeres of lampbrush chromosomes,[8] puffs occur at only about 2 per cent of the bands of giant polytene chromosomes.[3] Another difference between these two chromosomes is that the oöcyte chromosomes are destined to replicate after fertilization, whereas the polytene chromosomes of the dipteran larvae are in cells which will not divide and which will indeed be discarded at pupation.

Most interphase chromosomes are of course very much smaller than either lampbrush or dipteran polytene chromosomes. In the ordinary-size interphase chromosomes, DNA is the component which has been determined most often quantitatively. There is a well-known rule according to which the quantity of DNA per set of chromosomes is constant for the different cells of a given organism.[11, 12] The DNA content per set of chromosomes in somatic cells of the newt was determined in the work on which the constancy rule was established, and the constancy rule has led some investigators to assume that newt lampbrush chromosomes have the same DNA content. It may, however, be questioned whether the constancy rule, which applies fairly well to the chromosomes of somatic cells and sperm cells, holds for the strikingly different lampbrush chromosomes. In the generality of interphase chromosomes, as in the giant polytene chromosomes, most loci are relatively inactive; in lampbrush chromosomes, on the contrary, most loci are active. In this paper it will be shown that the DNA content per set of newt lampbrush chromosomes is in fact much higher than the DNA content per set of chromosomes in other cells of the newt. It will be shown also that the protein and RNA contents of lampbrush chromosomes are very different from those of the chromosomes in other cells.

Materials and Methods.—Ovarian eggs of the newt, *Triturus viridescens*, were used for all experiments on determination of chemical constituents.

Preparation of the whole nucleus: Procedures for dissecting the oöcytes were those developed by Duryee,[13] Gall,[7] and Callan and Lloyd.[8] A detailed description is given by Callan and Lloyd. Since it was recently found that newt oöcyte nuclei isolated in saline swell and lose dry weight,[14, 15] the medium for isolating nuclei to be used for DNA, RNA, and protein analyses contained 3% polyvinylpyrolidone (PVP) in 0.1 *M* NaCl adjusted to pH 6.8 with NaOH. In this medium an isolated nucleus was of the same size as a nucleus *in situ*. The cell membrane of the oöcyte in PVP solution was broken with the fine tip of tweezers under the dissecting microscope. The nucleus was squeezed out, and yolk platelets attached to the nuclear membrane were removed with the tweezers. After collection of a certain number of the nuclei into a conical 1-ml centrifuge tube using a fine-tipped pipette, PVP was washed out with cold absolute ethanol. The chemical analyses described below were done on this preparation.

Preparation of chromosomes: In this case nuclei were isolated in 0.1 *M* NaCl, pH 6.8 (*not* containing PVP), and nuclear membranes were peeled off with tweezers under the dissecting microscope, the membranes remaining attached to the tweezers. After a certain number of nuclei without their membranes were collected, they were transferred to the conical tube using a fine-tipped pipette. Nuclear materials were centrifuged down in an International Clinical Centrifuge. The supernatant was discarded and 0.5 ml of medium B of Callan and Lloyd[8] (0.1 *M* 5:1 KCl-NaCl, 1 vol, + 0.001 *M* KH_2PO_4, 4 vol) was added to disperse the nucleoplasm. Nuclear materials were stirred gently with a fine glass rod and then slowly centrifuged down at 50 × *g* for 2 min. The supernatant was again discarded, and the sediment washed once again in the same way. The packed materials were examined with an inverted microscope (a Zeiss "plankton" microscope) with phase contrast optics. The preparation consisted of lampbrush chromosomes and a few nucleoli.

DNA determination: Before chemical analyses of the chromosomes were undertaken, the ma-

terial was washed once with cold 5% TCA or 0.2 *N* PCA to remove acid-soluble materials. DNA was determined by a modified version of the fluorometric method of Kissane and Robins.[16] This method is far more sensitive than two others commonly used for DNA measurement, i.e., the Hoff-Jørgensen microbiological assay[17] and the Burton modification of the diphenylamine procedure.[18] The determination of DNA in the diphenylamine procedure and also in the fluorometric procedure of Kissane and Robins depends in the final analytical step on a reaction of desoxyribose—colorimetric in the former and fluorometric in the latter. Kissane and Robins showed that their method agreed well with the diphenylamine procedure for determination of DNA in rabbit brain tissue. We also compared values given by these two methods, using a newt liver homogenate, and got good agreement. Our experience is that when applied to the amphibian oöcyte, however, the fluorometric procedure gives a result for "DNA" about 10 times higher than does the diphenylamine procedure. This result was obtained using the Turner Fluorometer (and, with the exception to be mentioned presently, all of our determinations were done with this instrument) in which filters are used for both the exciting and emitted light. It seemed possible that measurement of the maximum wavelengths for excitation and emission might distinguish between DNA and other fluorescent material present in relatively high concentration in the oöcyte. These measurements were kindly made for us by Dr. Gerald Edelman. The maximum wavelengths were the same for both DNA and the fluorescent material of the oöcyte: 409 mμ for excitation and 532 mμ for emission. The component in DNA which fluoresces is the aldehydic group of desoxyribose, and the other fluorescent material in the oöcyte may well be an aldehyde.

For determinations of DNA in the nucleus and chromosomes of the newt oöcyte, the specificity of the procedure of Kissane and Robins was increased by using crystalline pancreatic DNAase. The sediment, in a conical centrifuge tube, of nuclear or chromosomal material isolated by the method mentioned above was treated with 0.5 ml of cold 5% TCA to remove acid-soluble materials and centrifuged down. After discarding the supernatant, the residue was washed with 0.5 ml of absolute ethanol once at room temperature and once at 60° C to remove lipids. Then one sample of the material was treated with 0.04 ml of DNAase-buffer solution (0.2 *M* phosphate buffer, pH 7.0, containing 0.003 *M* $MgCl_2$ and 0.4 mg DNAase/ml), and another sample was treated with 0.04 ml of the buffer-$MgCl_2$ solution for 5 hr at 37° C. At the end of the treatment the suspensions were centrifuged. Each supernatant was transferred to a conical tube using 50-μl micropipettes and dried in a centrifuge desiccator.[19] Fluorescence determinations were made on the residue in each tube. The DNA content of the material was taken to be the difference between the supernatant obtained by DNAase treatment and that simply after treatment with buffer. Calf thymus DNA was used as a DNA standard for fluorometry. The residues obtained after DNAase (or simply buffer) treatment and then centrifuging were washed twice with 80% ethanol, dried in the centrifuge desiccator, and the fluorescent values determined. The DNA content was taken to be the value of the residue after treatment with buffer minus the value of the residue after DNAase treatment. It can be seen from Table 1 that DNA determinations on the extracts and residues, as just described, check very well. It is also evident that if the material which is acted on by DNAase is taken to be DNA, most of the fluorescent material in nucleus and chromosomes is DNA. In contrast to the oöcyte cytoplasm, only 23–40% of the fluorescent material in the nucleus and only 10% of that in the chromosomes is not DNA.

DNA is a polynucleotide ordinarily containing a mixture of four or more different nucleotides. Whether the material we are determining is DNA in this sense is not known. The material we have determined is acid-insoluble, gives a reaction for desoxyribose, and is acted on by DNAase. At the present time there is no reason to consider the "DNA material" we have determined in newt oöcyte chromosomes to be essentially different in composition from the DNA ordinarily found in chromosomes.

RNA measurements: RNA contents of the nucleus and the chromosomes were calculated by subtraction of DNA content measured with fluorometry from total nucleic acid content measured by ultraviolet absorption. For total nucleic acid measurement, each material was first treated with cold 0.2 *N* PCA to remove acid-soluble materials. The residue was then extracted with 3:1 ethanol-ether at 60°C for 5 min to remove lipids. Finally, total nucleic acid was extracted in 0.2–0.3 ml of 0.5 *N* PCA at 70°C for 20 min, and measured by UV absorption at 260 mμ. Total nucleic acid content was calculated from the following formula: $E_{260}/ml = 3.3 \times 10^{-6}$ gm nucleic acid/ml.

Protein measurement: Protein analysis was done by the colorimetric procedure using the phenol reagent.[20] Isolated nuclei or chromosomes were treated with 5% cold TCA to remove acid-soluble materials. The residue was hydrolyzed with 0.1 ml of 0.6 *N* NaOH at room temperature for 3 hr. This hydrolysate was the starting material for colorimetry. Beef serum albumin (Armour) was used as a protein standard.

Results and Discussion.—(1) *DNA content of newt lampbrush chromosomes:* The result of the determination is given in Table 1. To compare the DNA content of

TABLE 1

DNA CONTENT OF NEWT OÖCYTE NUCLEUS AND LAMPBRUSH CHROMOSOMES

	(1) DNA equivalent in residue after being in buffer	(2) DNA equivalent in residue after DNAase treatment	(3) DNA content (1) minus (2)	(4) DNA in extract of buffer	(5) DNA in extract after DNAase treatment	(6) DNA content (5) minus (4)
Nucleus	(*a*) 2.5	(*a*) 1.0	(*a*) 1.5	0.5	1.8	1.3
	(*b*) 2.2	(*b*) 0.5	(*b*) 1.7	0.7	2.0	1.3
Chromosomes	0.88	0.10	0.78	less than 5×10^{-11}	0.65	0.65

All figures are grams $\times 10^{-9}$ per nucleus. 100 nuclei were used for each determination.

lampbrush chromosomes with that of the somatic chromosomes of *Triturus viridescens*, we take the value for this animal's erythrocyte nucleus given by Edström and Gall.[21] The erythrocyte nucleus is diploid, and the oöcyte at this stage is tetraploid.[22] To compare the DNA contents per set of diploid chromosomes, we take the DNA content of the erythrocyte nucleus and one half the DNA content of the chromosomes present in the oöcyte nucleus. From the figures given in the last column of Table 3, it can be seen that in lampbrush chromosomes the DNA content per chromosome set is more than four times that in erythrocyte chromosomes.

The DNA content per set of chromosomes in the erythrocyte is within narrow limits the same as that in other cell types of a given organism. This rule of constancy does not hold for lampbrush chromosomes. Since these are probably the only chromosomes which are active at all loci, it may be supposed that a large excess of DNA and activity are associated properties. The chromosomal activity which we are considering is the synthesis of RNA on a DNA template. Apparently, synthesis of RNA in lampbrush chromosomes is stepped up by increasing the number of DNA templates. This increase in DNA may well occur at active loci of other interphase chromosomes. If the percentage of active loci in them were about the same as the percentage of puffs (about 2 per cent) in the giant polytene chromosomes in Chironomus, the increase in DNA content of a set of ordinary interphase chromosomes would be approximately 10 per cent, hardly a significant departure from "constancy." Indeed the increase in DNA content in nuclei of the adrenal cortex observed in heightened cell activity is said to be about 10 per cent,[23] but the possibility has not been eliminated that this increase in DNA is not due to incipient mitosis.

The high rate of RNA synthesis in lampbrush chromosomes is associated with high DNA content, but there is no evidence for a continual replication of DNA. No incorporation of thymidine could be detected in autoradiographs made after exposing isolated oöcytes to labeled thymidine.[1, 2]

(2) *DNA in the nucleoplasm:* It can be seen from Table 1 that the nucleus contains twice as much DNA as is found in the chromosomes. There is as much DNA in the

nucleoplasm as in the chromosomes. Part of the nucleoplasmic DNA seems to be in the nucleoli. The evidence for this is as follows: the oöcyte nucleus in the lampbrush stage contains hundreds of nucleoli which are not attached to chromosomes, and because they are not attached, there has been some doubt whether these bodies are comparable to the nucleoli which in other nuclei are found attached to chromosomes, indeed to particular sites on chromosomes. In autoradiographic experiments with labeled RNA precursors (usually tritiated uridine), the RNA in free nucleoli of oöcyte nuclei, like that in the attached nucleoli of other nuclei, becomes intensely radioactive. This is prevented by actinomycin D,[2, 24] indicating that the process is DNA-dependent. Knowing that the oöcyte nucleoplasm contains DNA, one suspects that the DNA responsible for RNA synthesis in the nucleoli is located in the nucleoli themselves. It is, however, possible that the DNA is located elsewhere (perhaps in the chromosomes), whence the RNA passes to the nucleoli. In these experiments the labeled RNA precursors were supplied to isolated oöcytes. After RNA synthesis had occurred, the nuclei were dissected out and autoradiographs were made.

RNA synthesis in subnuclear organelles has been observed after removal of the nuclear membrane. The nucleus was first dissected out, the nuclear membrane torn off, and the gel-like nucleoplasm liquified and dispersed by adding salt solution C of Callan. Now chromosomes and nucleoli were not in an organized nucleus but merely present, and not associated, in the same salt solution. To promote RNA synthesis, the triphosphates of uridine, cytidine, guanosine, and adenosine (the last as H^3-ATP) were added. After 5 hr the material was fixed in acetic acid-formaldehyde, one preparation was treated with ribonuclease, and finally autoradiographs were made. In the preparation treated with ribonuclease nothing could be seen. In the others it was evident that RNA had been synthesized in the chromosomes and, in much higher concentration, in the nucleoli. If the synthesis is DNA-dependent, the experiment points to the presence of DNA in the nucleoli.

Presence of DNA in the nucleoli is a source of error in the determination of DNA in the chromosomes, for preparations of isolated chromosomes contain some nucleoli enmeshed in the chromosomal strands.

There have been no previous DNA determinations on the newt oöcyte nucleus. Finamore *et al.*[25] have reported very high DNA contents—several hundred times the tetraploid value—for the nuclei of mature frog oöcytes.

(3) *Protein, RNA, and DNA content:* Determinations of these components are given in Table 2. The ratios of protein to DNA and RNA to DNA are very much higher than in somatic nuclei and chromosomes. A comparison of these ratios for newt lampbrush chromosomes and the chromatin of beef liver is shown in Table 3.

The relative masses of newt lampbrush and newt liver chromosomes can be computed if it is supposed the protein:DNA ratio is the same in newt and beef liver chromosomes. Considering that lampbrush chromosomes have four times as much DNA as do newt liver chromosomes and that the ratio of protein to DNA is 200 times greater in lampbrush than in liver chromosomes, lampbrush chromosomes have 800 times the mass of liver chromosomes. The large amount of protein present in lampbrush chromosomes is at least in part the enzyme machinery needed for synthesis of RNA, a synthesis which is very active, considering the high RNA con-

TABLE 2

DNA, RNA, AND PROTEIN CONTENT OF THE NEWT OÖCYTE NUCLEUS AND LAMPBRUSH CHROMOSOMES

	Protein gm/nucleus	Total nucleic acid‡ gm/nucleus	DNA‡ gm/nucleus	RNA gm/nucleus	Protein/DNA	RNA/DNA
Nucleus	*14×10^{-7}	29×10^{-9}	1.5×10^{-9}	26.4×10^{-9}	1060	16.5
	17×10^{-7}	27×10^{-9}	1.7×10^{-9}			
	20×10^{-7}					
	Av.	Av.	Av.			
	17×10^{-7}	28×10^{-9}	1.6×10^{-9}			
Chromosomes†	3.3×10^{-7}	8.2×10^{-9}	0.78×10^{-9}	7.0×10^{-9}	550	9
	5.2×10^{-7}	7.3×10^{-9}				
		8.0×10^{-9}				
	Av.	Av.				
	4.3×10^{-7}	7.8×10^{-9}				

* 10 nuclei used per analysis.
† 20 nuclei used per analysis.
‡ 100 nuclei used per analysis.

TABLE 3

COMPARISON OF RATIOS OF PROTEIN:DNA AND RNA:DNA IN NEWT LAMPBRUSH CHROMOSOMES AND BEEF LIVER CHROMATIN

	Protein/DNA	RNA/DNA	DNA per diploid set
Beef Liver Chromatin	2.8	0.062	
Newt Lampbrush Chromosomes	550	9	0.39×10^{-9} gm
Newt Erythrocyte Chromosomes			0.089×10^{-9} gm*

* Value taken from paper by Edström and Gall (ref. 21).

tent and the rapid incorporation of labeled RNA precursors shown in autoradiographs.

The high RNA:DNA ratio of lampbrush chromosomes, about 100 times larger than the ratio for liver chromosomes, is of the right order, if it is supposed (by analogy with giant polytene chromosomes) that about 2 per cent of the loci are active in liver chromosomes, whereas all of them are active in lampbrush chromosomes. The RNA:DNA ratios of puffs and other regions in Chironomus polytene chromosomes were measured by Edström and Beermann.[26] They found that the puffs (active sites) had a higher RNA:DNA ratio than other parts of the giant chromosomes. High as this ratio was in the puffs, it was much lower than in lampbrush chromosomes. The ratio in the puff itself was probably higher than would appear from the analyses, for as the puff forms it extends over neighboring loci, so that when the puff is dissected out, neighboring, inactive loci are included.

The absolute amount of RNA in both nucleus and chromosomes given in Table 2 is greater than values reported by Edström and Gall.[21] One reason for the difference is that before analysis their preparations were fixed, and consequently some chromosomal loops may have been lost.

Summary.—The DNA content of lampbrush chromosomes in the newt oöcyte nucleus is about four times that of chromosomes in nuclei of other cell types in the newt. The nucleoplasm of the oöcyte contains as much DNA as is present in the chromosomes. Some of the nucleoplasmic DNA is located in nucleoli, which in this nucleus are not attached to the chromosomes. The protein:DNA and RNA:DNA ratios in lampbrush chromosomes are very much higher than in other chromosomes. The unusual characteristics of the composition of lampbrush chromosomes are related to the fact that these chromosomes, unlike others, are active at all loci.

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*VARIATION OF THE CONFORMATION OF THE ACTIVE SITE OF α-CHYMOTRYPSIN WITH HYDROGEN ION CONCENTRATION**

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It is common knowledge that α-chymotrypsin-catalyzed hydrolyses of many model substrates are inhibited by structurally related compounds incapable of functioning as substrates of this enzyme. It also is known that the *nature* of the inhibition process is dependent not only upon the structure of the inhibitor (modifier) molecule but also upon the structure of the substrate molecule.[1–3] We now find that for a given uncharged substrate-inhibitor (modifier) pair the *nature* of the inhibition (modification) process also is dependent upon the hydrogen ion concentration of the reaction system.

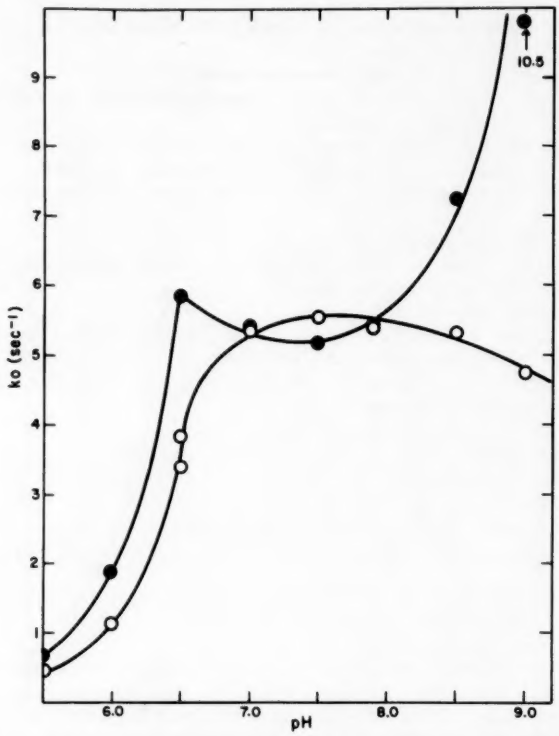

FIG. 1.—The α-chymotrypsin-catalyzed hydrolysis of acetyl-L-leucine methyl ester in aqueous solutions at 25.0° and 0.10 M in sodium chloride in the absence (open circles) and presence (closed circles) of benzamide as a function of the hydrogen ion concentration of the reaction systems.

In the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-leucine methyl ester, in aqueous solutions at 25.0° and 0.10 M in sodium chloride and from pH 5.50 to 9.00, the dependence of the rate of reaction upon enzyme and substrate concentrations, at any one hydrogen ion concentration, is given by equation (1).

$$-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S]). \quad (1)$$

The dependence of the kinetic parameter k_0 upon hydrogen ion concentration is described by the plot given in Figure 1. In these experiments $[E]$ varied between 0.544 and 1.655 μM[4] and [S] between 0.986 and 21.76 mM. The reaction was followed with the aid of pH-stat,[2] and the kinetic parameters k_0 and K_0 were evaluated with a Datatron 220 computer, as described previously.[5]

When the preceding reaction is examined over the same range of hydrogen ion concentrations but in the presence of benzamide, a known inhibitor of α-chymotrypsin-catalyzed reactions,[6,7] the dependence of the rate of reaction upon enzyme, substrate, and benzamide (inhibitor or modifier) concentrations at any one hydrogen ion concentration is described by equation (2),

$$-d[S]/dt = d[P]/dt = k_0[E][S]/\{K_0(1 + ([I]/K_I)) + [S]\}, \quad (2)$$

where $[I]$ is the inhibitor (modifier) concentration and K_I the associated kinetic parameter. The kinetic parameters k_0 and K_0 of equation (2) may or may not be equivalent to those of equation (1), depending upon the hydrogen ion concentration of the reaction system. In these experiments, conducted as described above, $[E]$ varied between 0.544 and 10.11 μM,[4] $[S]$ between 3.25 and 60.19 mM, and $[I]$ between 8.43 and 9.87 mM.

Comparison of the dependence of the kinetic parameter k_0 upon hydrogen ion concentration in the absence and presence of benzamide (cf. Fig. 1) demonstrates that the *nature* of the inhibition (modification) process varies with hydrogen ion concentration. Since both substrate and modifier are neutral molecules incapable of significant ionization within the range of hydrogen ion concentrations being considered, attention is necessarily directed to possible changes in the nature of the active site of the enzyme.

Fully competitive inhibition which arises from the formation of binary complexes of enzyme and substrate *or* enzyme and inhibitor demands that the kinetic parameter k_0 remain invariant in the absence or presence of inhibitor. It is seen from the plots given in Figure 1 that this condition is satisfied only in the region from pH 7.0 to 8.0 where the values of k_0, determined in the absence and presence of benzamide, are identical within the limits of experimental error. Thus, it is only in this region that the conformation of the active site of the enzyme is such as to lead to the formation of only binary complexes with the particular substrate and inhibitor being examined.

It does not follow that all substrates and inhibitors of α-chymotrypsin will form only binary complexes with the active site of the enzyme in the region from pH 7.0 to 8.0, since it is known that the consequences of ternary complex formation can be observed with structurally deficient substrates and inhibitors in this region.[1, 2, 8] However, it does appear that for model substrates of the type $R_1'CONHCHR_2$-CO_2R_3', where R_2 is larger than a hydrogen atom, binary complex formation will prevail only when the reaction system is between pH 7.0 and 8.0.

We can be further assured that with acetyl-L-leucine methyl ester and benzamide the inhibition is fully competitive in the region between pH 7.0 and 8.0 because, in addition to the invariance in the magnitude of the kinetic parameter k_0 noted above, we find a value of 5 $\pm$ 1 mM for the kinetic parameter K_I. This value is in reasonable agreement with that obtained previously[6, 7] from examination of systems in which nicotinyl-L-tryptophanamide was the substrate (K_I = 6.6 or 10 $\pm$ 2 mM at pH 7.9) or from systems in which acetyl-L-valine methyl ester was so employed[9] (K_I = 8.1 $\pm$ 0.9 mM at pH 7.9). A condition for fully competitive inhibition is that the value of K_I for a given inhibitor be independent of the structure of the substrate molecule.[10, 11]

Examination of the plots given in Figure 1 shows that above pH 8.0 benzamide no longer functions as a fully competitive inhibitor of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-leucine methyl ester. Instead, this compound now functions as an activator, increasing the magnitude of the kinetic parameter k_0 with decreasing hydrogen ion concentration of the reaction system and reversing the trend obtaining in the absence of modifier. Activation in the sense used above requires the formation of at least a ternary complex of enzyme, substrate, and activator (modifier). The phenomenon cannot be explained on the basis of binary complex formation. Thus,

with the substrate and modifier being neutral molecules incapable of significant ionization in the region of hydrogen ion concentrations under consideration, one is led to the conclusion that in passing from the region of pH 7.0 to 8.0 to higher pH values, the conformation of the active site changes from states where only binary complexes are allowed to those where at least ternary complex formation is feasible. The fact that the extent of activation is dependent upon the structure of both substrate and activator[9] leads us to the proposition that both species are in intimate contact with each other at the active site of the enzyme and that above pH 8.0 the active site of the enzyme is expanding with decreasing hydrogen ion concentration.

In the region from pH 7.0 to 6.5 the trend in values of the kinetic parameter k_0 appears to mirror that observed above pH 8.0. Again, there is evidence for activation through ternary complex formation and lessening significance of binary complex formation leading to fully competitive inhibition. The dramatic diminution in values of k_0 observed below pH 6.5 suggests the intrusion of another hydrogen ion dependent process which could be due to protonation of a group at the active site that is involved in the formation of reaction products, or possibly association of monomeric enzyme.[12, 13] While there is no question that between pH 6.0 and 7.0 the interaction of α-chymotrypsin with acetyl-L-leucine methyl ester and benzamide does not lead to fully competitive inhibition, the data given in Figure 1 could lead one to believe that this type of inhibition is again encountered at pH 5.5. However, this supposition is incorrect, as an extensive set of independent experiments conducted at pH 5.50, and presented in abridged form in Table 1, demonstrate that the presence of benzamide results in an enhancement in the magnitude of the kinetic parameter k_0 instead of the invariance demanded by fully competitive inhibition. Thus, we are led to the conclusion that from, at least, pH 5.50 to 7.0 and from pH 8.0 to 9.0, or greater, α-chymotrypsin interacts with acetyl-L-leucine methyl ester and benzamide to form at least ternary complexes and only in the mid-region from pH 7.0 to 8.0 are there formed binary complexes leading to fully competitive inhibition. Our interpretation is that on both the acid and alkaline sides of the region from pH 7.0 to 8.0 the conformation of the active site of the enzyme undergoes sufficient change, probably through expansion, to permit at least ternary complexes to be formed, whereas in the mid-region the conformation is such that only binary complexes are allowed with structurally sufficient substrates and inhibitors.

TABLE 1

MODIFICATION OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-LEUCINE METHYL ESTER AT PH 5.50 BY BENZAMIDE*

$[S]$ mM	$[E]$ μM	$[I]$ mM	$n - x$†	k_0‡ sec^{-1}	K_0‡ mM
1.13–9.02	21.2§	0.00	9–1	0.343 ± 0.006	5.07 ± 0.19
6.75–47.37	21.2§	9.24	10–2	0.527 ± 0.018	29.9 ± 1.9
1.10–8.81	21.2§	0.00	8–0	0.410 ± 0.032	8.18 ± 1.03
6.60–46.23	21.2§	9.06	7–0	0.531 ± 0.029	29.3 ± 3.1
2.72–21.76	1.66**	0.00	14–2	0.470 ± 0.010	6.47 ± 0.34
8.60–60.19	10.1**	8.43	8–0	0.670 ± 0.040	35.4 ± 4.0

* In aqueous solutions at 25.0° and 0.10 M in sodium chloride.
† n = no. of experiments; x = no. rejected by computer program.[5]
‡ Kinetic parameters of equation (1).
§ Crystalline bovine α-chymotrypsin, Worthington Lot CDI 6066–67.
** Crystalline bovine α-chymotrypsin, Armour Lot T 97207.

Experiments with acetyl-L-leucine methyl ester and formanilide, and acetyl-L-valine methyl ester and benzamide, have yielded results which are qualitatively similar to those described above.[9] Since all of the experiments have revealed a situation far more complex than originally anticipated, we believe it premature to attempt a quantitative analysis of the dependence of the modification process upon hydrogen ion concentration at this time. It is evident that all previous attempts to explain hydrogen ion dependencies of kinetic parameters encountered in α-chymotrypsin-catalyzed hydrolyses on the basis of localized ionization phenomena associated with an active site of invariant conformation will have to be re-examined in the light of the results reported in this communication. It also follows that interpretative extrapolations over a wide range of hydrogen ion concentrations must be viewed with added caution. Finally, it is evident that the discovery of the multiple dependence of the nature of the modification process upon the structure of substrate, modifier, and hydrogen ion concentration of the reaction system has provided a new and useful tool for determining the mechanism of α-chymotrypsin-catalyzed reactions.

Summary.—It has been shown that the nature of modification (inhibition or activation) processes encountered in α-chymotrypsin-catalyzed reactions is dependent upon the hydrogen ion concentration of the reaction system. This observation has been interpreted in terms of variation of the conformation of the active site of the enzyme with hydrogen ion concentration.

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† Contribution no. 3029.

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*STRUCTURAL PROTEINS OF MEMBRANE SYSTEMS**

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The mitochondrion may be conceived of as a two-layered system to which are attached many thousands of macromolecular particles collectively referred to as elementary particles.[1] The two layers or membranes are made up of a matrix material containing both protein and lipid.[2] We further postulate that the ele-

mentary particles are of two types: (1) electron transfer particles that contain the respiratory chain; (2) ancillary particles that contain the enzymes implicated in citric acid cycle oxidations, β-oxidation of fatty acids, lipid biosynthesis, etc.[2] Our primary concern in the present communication is with the nature of the protein in the mitochondrial membrane system to which the elementary particles are attached and with the evidence for the presence of similar proteins in other membrane systems.

The electron transfer particle can be resolved into four component complexes.[3-6] Two observations were made during the course of these fragmentation studies that deserve special attention here. The first concerns the nature of the reagents necessary for the splitting of the chain into the individual complexes. Lipophilic reagents have been found to be most efficacious for this purpose, and the effectiveness of this type of reagent in fragmenting the mitochondrion was a primary reason for proposing that hydrophobic bonding was of importance in protein-protein and protein-lipid interactions in the mitochondrion.[7] The second observation was that in the isolation of the oxidation-reduction protein components of the respiratory chain the initial separations of the components were relatively easy and complete, but the further purification of the proteins required the removal of a tightly bound, colorless, water-insoluble protein. This colorless protein material appeared to be the major impurity present. The known oxidation-reduction components of the electron transport particle account for only a small percentage of the total protein of the mitochondrion. These considerations led to the proposal by Green *et al.*[8] of the existence of structural protein which is primarily concerned with the structural integrity of the mitochondrion. Further, it was proposed that the bonds between structural protein molecules, as well as those between structural protein and lipids, were hydrophobic in nature.[9]

One of the properties of structural protein was the ability to combine with phospholipid in the form of a complex. The original preparation of Green *et al.*[8] interacted with phospholipid[10] to a significant degree only under alkaline conditions. However, structural protein can be prepared in a form[9] that is capable of combining with phospholipids almost maximally at neutral pH. Furthermore, the lipid-binding behavior of this preparation of Richardson *et al.*[9] corresponds closely with that of an acetone-extracted mitochondrial preparation (the "10% preparation" of Fleischer *et al.*[11]). The mechanism of the interaction with phospholipid of the Richardson preparation of structural protein has been studied in some detail, and the available evidence suggests that the primary bonds involved in the interaction are indeed hydrophobic in nature.[12] In addition, it has been shown that the structural protein prepared in this way is capable of binding inorganic phosphate and certain phosphate esters such as ATP and DPNH.[13]

Our present concept of membrane structure involves, then, a backbone of structural protein which is primarily responsible for the integrity of the membrane. To this is attached the lipid which is bound to the structural protein chiefly through hydrophobic bonds[12] and in a micellar form.[14] That it is the structural protein and not the phospholipid that is primarily responsible for the integrity of the beef heart mitochondrial membrane has been shown conclusively by Fleischer *et al.*[11] who were able to extract up to 80 per cent of the mitochondrial phospholipid (in more recent

experiments up to 92%) without affecting the structural integrity of the mitochondrion as judged by electron microscopy.

If the concept of the "structural protein" has general applicability to membrane systems, it should be possible to isolate a similar protein from other membrane systems. One of the difficulties in the isolation and characterization of a structural protein is the paucity of specific criteria by which the protein can be identified and recognized as such. The property of water insolubility at neutral pH and the ability to bind phospholipids are the most diagnostic. We have also found that the binding of phospholipid by structural protein of beef heart mitochondria is affected by the pH of the medium and by modification of the protein.[9] For example, if the reaction medium is made alkaline or if urea is added in high concentration, the binding of lipid by the protein is greatly increased. If the protein is first brought to an alkaline pH and the pH is then readjusted to neutrality before addition of the phospholipid, a small but consistent increase in binding is noted. The rationale of these procedures will be discussed elsewhere.[9] Suffice it to say that these studies have provided additional criteria by which to identify structural protein. Further, the binding of inorganic phosphate or phosphate esters as demonstrated with the structural protein of beef heart mitochondria[13] may be a general characteristic of structural protein as a class. With these considerations in mind we attempted to isolate a "structural protein" from several different membrane systems.

Methods.—The phospholipid used in our studies was Asolectin (a commercial preparation of soybean phospholipid, product of Associated Concentrates, Woodside, L. I.). A suspension was prepared by homogenization and sonication of the crude mixture in 0.02 *M* Tris-acetate buffer of pH 8.0 containing 0.001 *M* EDTA.[28] ATP was purchased from Sigma Chemical Co., St. Louis, and was used as the K^+ salt. The methods used in carrying out the reactions and analyses will be described in detail elsewhere.[9] Briefly, structural protein (5 mg) was treated with the phospholipid (10 mg) prepared as described above. After a 15-min incubation period at room temperature the insoluble protein-lipid complex was removed by low-speed centrifugation and washed to remove excess lipid. Phospholipid was determined as total phosphorus by a modification of the procedure of Chen *et al.*,[15] and protein was determined by the biuret technique of Gornall *et al.*[16] In the ATP-protein complex formed by the treatment of the structural protein (5 mg) with ATP (10 μmoles) in a total volume of 1 ml,[13] the ratio of the components was determined in a similar manner, viz., by the determination of total phosphorus and of protein.

Isolation of membrane systems: In all the procedures described below, the temperature was maintained at 0–5°C.

(1) *Beef heart mitochrondria:* Heart mitochondria were prepared as described previously.[17]

(2) *Beef liver mitochondria:* 400 gm of fresh beef liver were chopped up and suspended in 800 ml of 0.25 *M* sucrose which was 0.05 *M* in Tris-acetate buffer of pH 7.4. The juice was squeezed out through cheesecloth, and the solid material was resuspended in the sucrose-Tris buffer and homogenized. The suspension was then centrifuged for 20 min at 600 × *g* and the residue was discarded. The supernatant fluid was decanted through cheesecloth and centrifuged for 15 min at 10,000 rpm in the No. 30 rotor of a Spinco Model L ultracentrifuge. (This supernatant fluid was used in the preparation of microsomes.) The residue was taken up in a buffered mixture which was 0.25 *M* in sucrose, 0.05 *M* in Tris-acetate, pH 7.4, and 0.001 *M* in EDTA; the mixture was homogenized and sedimented for 10 min at 17,000 rpm in the No. 30 rotor. The residue was taken up again in the sucrose-Tris-EDTA buffer; the mixture was homogenized, diluted with $\frac{1}{2}$ its volume of 0.9% KCl, and stored in the frozen state.

(3) *Beef liver microsomes:* The supernatant fluid (specified in the preceding section for use in the preparation of microsomes) was recentrifuged in the No. 30 rotor for 30 min at 30,000 rpm. The reddish pellet was discarded, and some floating yellowish particles were removed by straining through glass wool. The supernatant fluid was then centrifuged for 2 hr at 40,000 rpm in the

No. 40 rotor of the Spinco ultracentrifuge. This pellet was taken up in the sucrose-Tris-EDTA buffer; the mixture was homogenized, diluted with 0.9% KCl, and frozen until ready for use. Difference spectra of this microsomal preparation in its oxidized and reduced forms (dithionite reduction) gave absorption maxima at 556, 526, and 425 mμ, wavelengths which are characteristic of the microsomal cytochrome b_5.[18]

(4) *Bovine erythrocyte stroma:* Fresh beef blood was collected in a citrate-dextrose solution (ACD preservative, National Institutes of Health, Formula A), and the mixture was immediately chilled. The corpuscles were collected and washed 3 times by centrifugation with 0.9% NaCl; the supernatant fluid and buffy coat were removed by aspiration. The cells were suspended for 30 min in isotonic phosphate buffer (approximately 0.01 *M*) at pH 8.0, collected by centrifugation, lysed with dilute phosphate buffer, and the stroma obtained by centrifugation was washed essentially free of hemoglobin with the same buffer according to the method of Dodge *et al.*[19] Alternatively, the stroma was prepared by the method of Parart[20] in which packed and washed red blood cells were lysed with 1 vol of distilled water and the lysate was treated with 80 vol of CO_2-saturated water. Under these conditions the stroma protein is agglomerated and can be isolated by centrifugation. Successive washings with CO_2-saturated water remove most of the hemoglobin.

(5) *Spinach leaf chloroplasts:* The "green precipitate" fraction of spinach leaf chloroplasts was prepared according to the procedure of Park and Pon.[21] Chloroplasts isolated by differential centrifugation of homogenized spinach leaves were sonicated and centrifuged. The floating lipid-containing layer and the light-green-colored supernatant fluid were removed by aspiration; the remaining "green precipitate" which corresponds in size and structure to the lamella structures of the intact chloroplast[21] was collected for use.

Preparation of structural proteins: Structural protein was prepared from the membrane systems of beef liver mitochondria and microsomes, and from the stroma of red blood cells by a modification[9] of the method of Green *et al.*[8] originally applied to beef heart mitochondria. The modified procedure is described elsewhere in detail;[9] no significant changes were required for any of the above preparations. It involves a "solubilization" of the material with cholate and deoxycholate followed by a fractional precipitation of the structural protein with ammonium sulfate. The precipitate formed at 0–0.12 saturation at 4° is collected. The detergents are removed by a modification of the acetone extraction procedure of Lester and Fleischer.[23] The removal of detergents is essential for achieving maximal binding of phospholipid.

Cholate and deoxycholate were not effective in "solubilizing" the green precipitate of the spinach chloroplasts. This material with no further purification was therefore extracted with aqueous acetone[23] to remove most of the phospholipid. The bulk of the chlorophyll was also extracted by acetone. The erythrocyte stroma was similarly treated to permit comparison of binding properties between proteins prepared without detergent and protein prepared with detergent.

Results—All of the preparations described bind significant quantities of phospholipid at neutral pH. A summary of the capacity of the protein in the several preparations to bind phospholipid and ATP is given in Table 1. Although the exact numerical values vary, certain consistencies may be seen among the different preparations. In all cases there is an increase in the phospholipid bound when the reaction is carried out at pH 12 compared to that bound at pH 7, even when the pH of the medium was readjusted to 7 before the lipid-protein complex was sedimented. The same type of effect was noted when the reaction medium was 8 *M* in urea. The degree of each of these effects varied considerably and is probably a reflection of variations in the chemistry of the structural proteins. Richardson *et al.*[9] have proposed that the efficacy of high pH and of urea in increasing the binding of phospholipid is an expression of an unmasking of additional binding sites as a result of conformational changes induced by these reagents (high pH or urea) or of monomerization of the structural protein (high pH). When the pH of the protein suspension was adjusted to 12 and then brought back to neutrality before the addition of phospholipid, a small but significant increase in the binding was noted in all the

TABLE 1

BINDING OF PHOSPHOLIPID AND ATP TO VARIOUS STRUCTURAL PROTEIN PREPARATIONS

Condition	Starting material (μg P per mg of protein)	Phospholipid phosphorus (μg P per mg of protein*)					ATP phosphorus, μg P per mg of protein§
		pH 7	pH 12†	pH 12 → 7‡	8 *M* urea	1 *M* KCl	
Source of material							
Beef heart mitochondria	0.28	10.2	36.3	13.8	16.8	8.1	4.0
Liver mitochondria	0.37	6.8	10.9	11.3	14.8	7.7	2.8
Liver microsomes	0.35	7.2	12.0	8.1	10.2	6.0	5.3
Bovine red blood cells							
Detergent-treated	0.10	4.2	15.2	4.5	14.1	4.3	0.2
Acetone-extracted							
Hanahan[19] preparation	5.0	10.8	16.4	16.2	31.9	18.3	...
Parpart[20] preparation	4.1	7.9	24.6	14.9	22.0	17.0	0.3
Spinach leaf chloroplasts							
Acetone-extracted green residue	1.2	3.4	11.7	6.5	10.2	11.1	2.2

* Phospholipid (Asolectin) was incubated with protein on a 2:1 weight basis for 15 min at room temperature. The complex was removed by centrifugation, the pellet washed twice, and the ratio of phosphorus to protein determined.

† The mixture was incubated at pH 12 and neutralized before removing the complex from solution.

‡ The protein was taken to pH 12 and then readjusted to pH 7 before the additionof phospholipid.

§ ATP (10 μmoles) was incubated with 5 mg of protein and 40 μmoles of Tris-acetate buffer, pH 7, for 10 min at room temperature. The complex was centrifuged, washed, and the phosphorus-to-protein ratio determined.

preparations except that of the red blood cell stroma prepared with detergents. The increases in this case were not as great as when the reactions were carried out at the high pH. One possible explanation for the increase induced by exposure to alkali would be in terms of a rearrangement of the protein polymer after depolymerization at the high pH and repolymerization at neutral pH. This rearrangement could expose additional binding sites.[9] The effect of 1 *M* salt on the binding of phospholipid by structural protein is complicated. The effect of salt on the binding of phospholipid by structural proteins prepared with detergents is not consistent. Salt, on the other hand, has always greatly increased the uptake of phospholipid by the acetone-extracted protein of chloroplast and of the red blood cell membrane that had not been exposed to detergent.

The structural proteins prepared from beef heart mitochondria, beef liver mitochondria and beef liver microsomes, and from the acetone-extracted green precipitate of spinach leaf chloroplast were all capable of binding considerable amounts of ATP.[13] The structural protein of the red cell stroma, whether or not exposed to detergents, bound only minimal amounts of ATP under the conditions used.

Although the identification of a protein as a structural protein is a difficult task, primarily because satisfactory criteria for characterization are lacking, nevertheless the experimental results presented in this communication establish the fact that proteins isolated from several membrane systems (i.e., liver and heart mitochondria, liver microsomes, erythrocyte stroma, and the lamellar structures of leaf chloroplasts) all show qualitatively similar properties in the binding of phospholipid over a wide range of conditions, although the quantitative results vary from preparation to preparation. The close similarity in binding properties of these proteins suggests that we may be dealing with a general class of protein.

One property requisite for a role as a structural element is insolubility under physiological conditions. All of the protein preparations examined were extremely water-insoluble at neutral pH in the absence of solubilizing agents such as sodium

dodecyl sulfate.[24] Salt appeared to decrease the solubility further. Even under conditions by which structural protein could be solubilized, e.g., pH 12 in the case of the mitochondrial and microsomal preparations, the effect of salt was to drive the protein out of solution[22]—an observation that reinforces the contention that it is primarily hydrophobic bonds that are responsible for the interaction of the monomers of structural protein in an aqueous environment.[7] This type of bond should give great stability to a polymeric array of the protein in membranes.

We have succeeded in preparing water-insoluble proteins from several membrane systems with similar capacities for binding phospholipid and ATP. We propose that this type of protein is responsible for the maintenance of the structural integrity of the membrane. If extrapolation can be made from conclusions arrived at by studies on the structural proteins of beef heart mitochondria,[25] it is probable that the structural proteins form a network system with phospholipid that makes up the matrix of the membrane layer. The elementary particles associated with the membrane system are probably attached to this network by stalks of the kind observed for the elementary particles of the mitochondrion.[24, 27, 29]

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*THE UNFOLDING AND RENATURATION OF A SPECIFIC UNIVALENT ANTIBODY FRAGMENT**

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The chemical basis for antibody specificity has not, so far, been determined. Three major possibilities exist.

(1) The large variety of specificities is generated by differences in amino acid sequence in some portion of the antibody molecule.[1, 2] Each molecule with a given sequence forms a unique set of disulfide bonds, and folds to a unique conformation under physiological conditions.

(2) The specificities are generated predominantly by the arrangement of disulfide bonds.[3] Antibody molecules which differ in primary structure certainly exist (genetic variants, for example), but the number of possibilities is much too small to account for the number of possible specificities. However, each primary structure may lead to a large number of distinguishable molecules by formation of different sets of disulfide bonds. Once the location of disulfide bonds is fixed, each molecule folds to a unique conformation under physiological conditions.

(3) The specificity is generated predominantly by noncovalent bonds.[4] Molecules with a given primary structure and pattern of disulfide bonds may be folded into numerous possible conformations. In the presence of antigen (at the site of biosynthesis) a complementary conformation is energetically favored, and this is maintained under physiological conditions.

In trying to distinguish between these possibilities, one is hampered by the fact that even highly specific antibody preparations are always chemically heterogeneous. One method exists which circumvents this difficulty. This method involves deliberate destruction of the specific conformation, followed by attempts to regenerate specific activity. A number of studies using this approach have been reported, the most elegant being the work of Karush.[3] His procedure was to destroy the specific conformation and biological reactivity of an anti-hapten antibody by the action of 8 *M* urea. Upon dialysis to remove the urea, the antibody protein returned to a globular form and regained almost all of its original activity. This experiment would seem to eliminate possibility (3), since that mechanism for generating antibody specificity would require the presence of antigen (and, presumably, the antibody-forming cell) to produce the biologically active conformation.

Karush also showed that antibody activity can be destroyed by breaking disulfide bonds. He speculated that this might mean that possibility (2) for the chemical

origin of antibody specificity is the correct one. No supporting evidence, however, exists. No attempt was made to recover activity by allowing the broken disulfide bonds to reform. If antibody activity could be recovered in this way, possibility (2) would be eliminated.

For two reasons it has seemed desirable to us to repeat the essential features of Karush's experiment. Firstly, there is a question (Nisonoff and Pressman[5]) as to whether 8 *M* urea in fact disrupts the noncovalent structure of the antibody combining site. This question is particularly pertinent in view of the finding of Winkler and Doty[6] that antibody activity cannot be recovered after five days' exposure to 10 *M* urea at room temperature. However, their experiments are themselves open to question because chemical changes other than a simple unfolding of the noncovalent structure may have occurred under the conditions they used.[7]

The second reason for reinvestigating the problem is the important discovery of Porter[8] that papain acts on antibody protein to produce univalent fragments which have the same specificity and quantitatively the same affinity for antigen[9] as the parent antibody. This makes possible a study of renaturation on a molecule which is only one third the size of the parent protein, and contains fewer than one third of the original disulfide bonds.

Materials and Methods.—Rabbit antibody to bovine serum albumin was prepared by the method of Singer *et al.*[10] Sixty eight per cent of it was found to be precipitable with specific antigen. Univalent active fragment (fragment I) was prepared from the antibody protein by the method of Porter.[8] By two different methods (see below), 63% of the product was found capable of combining with specific antigen. Fragment I was also obtained[11] from a sample of nonspecific rabbit γ-globulin, prepared from pooled rabbit sera by repeated precipitation with $(NH_4)_2SO_4$ at a concentration of 250 gm/liter. While this procedure does not lead to very pure γ-globulin, it should be noted that the preparation of fragment I from the γ-globulin involves a chromatographic separation which provides a specific purification step.

Bovine serum albumin was obtained from Sigma Chemical Co. Its conjugate to polystyrene was prepared by the method of Gyner and Sehon,[12] using polyaminopolystyrene purchased from Norsk Hydrelectrik Co. (Oslo, Norway).

Guanidine hydrochloride was prepared from Eastman guanidine carbonate by the method of Anson.[13] Other chemicals were of reagent grade.

Protein concentrations were determined from measurements of absorbance at 278 mμ. The absorbance of fragment I, at a concentration of 1 gm/100 cc for a light path of 1 cm, was obtained from a solution whose concentration had been determined by drying to constant weight at 107°C. The value found was 15.0.

Sedimentation velocities were measured with a Spinco analytical ultracentrifuge. Most measurements employed schlieren optics, though interference optics were used in one experiment. Most measurements were made at 59,780 rpm, at 25°C. Those shown in Figures 2 and 3 used two double-sectored cells in the same rotor, one cell containing a solution of antibody fragment I and serum albumin in one sector and solvent in the other. The other cell was similar, except that inactive fragment I was substituted for antibody fragment.

Optical rotation measurements were made with a Rudolph spectropolarimeter equipped with oscillating analyzer, and with a thermostatically controlled cell compartment. Specific rotation measurements were converted to reduced mean residue rotations[14] by the relation

$$[m']/[\alpha] = 3\,M/100(n^2 + 2)$$

where M is the mean residue weight of the protein, taken to be 115 for fragment I, and n is the refractive index of the solvent. Values of n for guanidine hydrochloride solutions, at 589 mμ, were taken from Kielley and Harrington.[15] Their wavelength dependence was estimated from an empirical procedure in use in this laboratory. Values of n for urea solutions, at 365 mμ, were obtained from Schellman.[16]

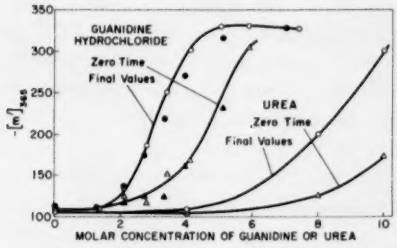

FIG. 1.—The unfolding of nonspecific fragment I by guanidine hydrochloride, as measured by optical rotation, at a protein concentration of 0.15–0.5 gm/100 cc, pH 5.5, and 15°C. Open circles and triangles represent data obtained with fragment I. Filled circles and triangles were obtained with a fragment I which had been fully unfolded (at 5°C) and then refolded by slow removal of guanidine. The corresponding data with urea were determined under similar conditions, at 25°C.

Results.—Various studies now in progress in this laboratory indicate that γ-globulin and fragment I have native globular conformations of high stability. Only guanidine hydrochloride, of the reagents studied so far, is able to produce complete unfolding. The results of optical rotation studies of the unfolding of fragment I from a nonspecific γ-globulin sample, using guanidine hydrochloride as unfolding agent, are shown in Figure 1. The reaction is time-dependent and follows complex kinetics which we have not attempted to analyze. The figure shows two curves, one resulting from the extrapolation of experimental data at each concentration of guanidine to zero time, the other representing final values which remained unchanged over a period of one hour or more. Up to 20 hr were required to attain these final values at the lower guanidine concentrations, but 2 hr sufficed at concentrations of 6 *M* or above.

The data show that at sufficiently high concentrations of guanidine the reaction goes to "completion," in the sense that no further change occurs with addition of more guanidine or longer time of exposure. The equilibrium curve (final values) is a typical protein unfolding curve, and there is every reason to believe that the absence of further change at guanidine concentrations above 6 *M* indicates that no noncovalent bonds remain and that the protein is as completely unfolded and randomly coiled as its disulfide bonds permit. It should be noted, in support of this conclusion, that the final value of $[m']_{365}$ is similar to the value of the same parameter observed for several other completely unfolded proteins.[14] Analysis of optical rotation data at other wavelengths by the Moffitt-Yang equation[14] leads to a small positive value for the parameter b_0, and a value in the range of $-650°$ to $-700°$ for the parameter a_0, when λ_0 is set equal to 212 mμ. These values are again characteristic of completely unfolded proteins.[14] Similar evidence is provided by experiments with the ultracentrifuge.[17] Sedimentation equilibrium studies of fragment I yield the same molecular weight in 6 *M* guanidine as in dilute salt solutions, but the second virial coefficient in guanidine is 3×10^{-4} (cc/gm) (gm/mole)$^{-1}$, which is an order of magnitude larger than for a typical globular protein. The sedimentation coefficient $s_{20,w}$ determined in 6 *M* guanidine is 1.95×10^{-13}, compared to the value of 3.7×10^{-13} observed in dilute salt solutions.

Figure 1 shows that similar experiments in urea solutions do not lead to complete unfolding. Since we have found a close similarity between the unfolding of intact γ-globulin and of fragment I in this regard, the results indicate that complete unfolding probably was not achieved in the experiments of Karush[3] cited earlier.

Reversal of the unfolding of the protein was brought about by the slow removal of guanidine by dialysis over a period of several hours at 5°C. In preliminary experi-

ments, in which the dialysis was carried out at 25°C, the final solution was slightly turbid, and a small quantity of protein was precipitated on heating to 40°C and removed by centrifugation. When dialysis was carried out at lower temperatures, the solutions were also heated to 40°C and centrifuged, but no visible precipitate was found. (It should be noted in this connection that the unfolding reaction itself shows little dependence on temperature in the range of 4–25°C.)

The refolded material proved to be virtually indistinguishable from native fragment I. It gave a single sharp peak in sedimentation velocity experiments, and its optical rotatory properties were the same as those of the native protein. Most convincing is a repetition of the unfolding by guanidine hydrochloride, shown as filled circles and filled triangles in Figure 1. The data are seen to differ only very little from those obtained with the original native protein. It is evident that the unfolding brought about by guanidine can be essentially completely reversed, and the original globular conformation essentially regained.

To demonstrate that the denaturation of specific antibody fragment I follows a course similar to that observed for the nonspecific fragment, rotation measurements were carried out on solutions of the antibody fragment at zero, 6.3 M, and 7.3 M guanidine hydrochloride. The final rotation values at 365 mμ were $[m'] = -105°$ $-331°$, and $-336°$, respectively, in complete accord with the data of Figure 1. This shows that the unfolding of the biologically active protein, like that of inactive fragment, is complete at high guanidine concentrations.

The antibody fragment was unfolded and refolded in the same way as nonspecific fragment I. In one experiment it was exposed to 6 M guanidine for 2 hr; in a second experiment it was exposed to 7.3 M guanidine for 12 hr. After dialysis to remove guanidine and heating to 40°C, a small amount of insoluble material was observed in the second experiment. This was removed before activity tests were carried out. In the first experiment no insoluble material appeared.

The ability of fragment I to combine with antigen (bovine serum albumin) was measured in the ultracentrifuge. Fragment I and serum albumin have closely similar sedimentation coefficients ($s_{20,w} = 3.5$, and 4.4×10^{-13}, respectively). A mixture of nonspecific fragment I and bovine serum albumin (molar ratio about 5:1) gave a single peak (Fig. 2) with a sedimentation coefficient of 3.5×10^{-13}, and a barely detectable shoulder on the fast side. A similar mixture of native antibody fragment I and bovine serum albumin, however, is seen from Figure 2 to have two peaks, the faster one having the larger area, and moving with a sedimentation velocity corresponding to $s_{20,w} = 9.2 \times 10^{-13}$. The latter figure is approximately the expected sedimentation coefficient for a tight complex containing about 2.5 fragments I per albumin molecule, if it is assumed that $s_{20,w}$ increases as (molecular weight)$^{2/3}$. The slower peak has $s_{20,w} = 3.5 \times 10^{-13}$ and presumably consists entirely of unreacted fragment I.

The relative areas under the peaks of such a sedimentation diagram cannot be rigorously used to measure the relative concentrations of complex and unreacted molecules, because complex formation is reversible and dissociation may occur as the complex sediments away from unreacted fragment I.[18] There are features of the reaction observed here which suggest that such dissociation does not occur to a significant extent, at least with native protein. But the question is, in any event, not of primary importance, because our principal purpose is to compare native

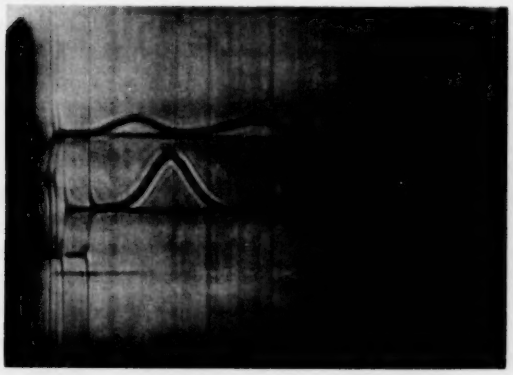

FIG. 2.—Sedimentation diagram of native antibody fragment I in the presence of bovine serum albumin (upper trace). The lower trace is a control experiment, showing native nonspecific fragment I in the presence of bovine serum albumin. The rotor speed was 59,780 rpm, and the picture was taken 48 min after full speed was reached. The peak of the lower trace moves with the same velocity as the slower peak of the upper trace. It appears at a different location because the menisci are in different positions.

antibody fragment I with its refolded counterpart and to see whether substantially similar results are obtained with both proteins. Some uncertainty in the quantitative measure of the extent of combination is permissible.

We have accordingly assumed that the area under the faster peak represents all of the bovine serum albumin originally added and all of the fragment I capable of reacting with it. The slow peak is taken to measure the content of unreactive fragment I. It is found in this way that 63 per cent of the native fragment I can combine with the specific antigen, a figure comparable to the 68 per cent precipitability of the antibody protein from which the fragment was derived.

Figure 3 shows data similar to those of Figure 2, obtained with the refolded antibody fragment. Similar estimates of antibody reactivity were made, and the results of two independent experiments of this kind are shown in Table 1. It is evident

TABLE 1

MEASUREMENT OF SPECIFIC COMBINATION WITH ANTIGEN BY SEDIMENTATION VELOCITY

Fragment I	Sed. coeff. ($s_{20,w}$ × 10^{13})* Slow peak	Fast peak	Per cent of total area† under slow peak	Per cent of fragment I Combined with antigen	Renatured
Native	3.3	9.2	31	63	—
Refolded‡	3.4	8.4	45	46	73
Native	3.6	9.2	30	63	—
Refolded§	3.4	7.9	39	51	82

* Measured with a precision of about 0.2.
† After correction for radial dilution, the sum of the areas under the two peaks was the same as the area under the single peak in the control experiment. In calculating the per cent reactivity of fragment I from the figures in this column, the part of the total area ascribable to serum albumin has been subtracted.
‡ After two-hr exposure to 6 *M* guanidine.
§ After 12-hr exposure to 7.3 *M* guanidine. These are the data shown in Figs. 2 and 3.

that 70–80 per cent of the refolded molecules have retained the ability to combine with antigen. To show that this represents a specific reaction, a sedimentation run was performed, using refolded fragment I and human serum albumin rather than bovine. A fast-moving complex could not be detected. Bovine serum albumin was then added to the same solution, and the same fast peak was observed as in the earlier experiments.

Two minor aspects of the sedimentation data obtained with refolded fragment I should be noted. (1) The sedimentation coefficient of the complex was always smaller than in the corresponding experiment with native fragment. This is the

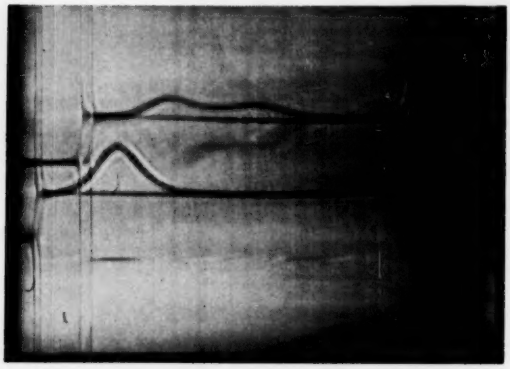

FIG. 3.—Sedimentation diagram of renatured antibody fragment I in the presence of bovine serum albumin (upper trace). The lower trace is a control experiment showing renatured nonspecific fragment I in the presence of bovine serum albumin. Rotor speed was 59,780 rpm, and the picture was taken 44 min after full speed was reached. As in Fig. 2, the peak of the lower trace moves with the same velocity as the slower peak of the upper trace.

expected result, since the average serum albumin molecule must be combined with fewer fragment I molecules if 20–30 per cent of the latter have become inactivated. (2) The refractive index gradient between the two peaks in Figure 3 does not fall to zero, although it does so in the corresponding experiment with native fragment. This could mean that some dissociation of the complex occurs at the low concentration side of the fast-moving boundary,[18] thereby implying that some of the renatured protein possesses a reduced affinity for antigen. However, alternative explanations exist.

A second method for determining the ability of fragment I to combine with antigen was to measure the extent of adsorption on a polystyrene resin to which bovine serum albumin had been covalently coupled. The extent of adsorption was measured by allowing the protein to stand for 30 min in contact with the conjugated resin on a filter. The uncombined protein was then washed through the filter with a buffer solution, the total volume collected being ten times the volume of the original protein solution. The optical density of the filtered solution was then compared to that of the original protein solution, diluted 1:10 with buffer. The results are reported in Table 2. A little uncertainty is introduced by the fact that some nonspecific adsorption is observed even when nonspecific fragment I is used, but the

TABLE 2

MEASUREMENT OF SPECIFIC COMBINATION WITH ANTIGEN BY SORPTION TO POLYSTYRENE-COUPLED SERUM ALBUMIN

	Optical Density at 278 mμ		Per cent of Fragment I		
Fragment I	Original solution	After filtering through resin-bound serum-albumin	Adsorbed to resin	Specific active	Renatured
Native nonspecific	0.152	0.134	12	0	—
Refolded nonspecific	0.144	0.106	27	0	—
Native anti-BSA	0.154	0.050	68	64*	—
Refolded anti-BSA	0.154	0.065	58	42†	66‡

* Assuming that 12% of the protein is still nonspecifically adsorbed, this percentage is based on the remaining 88%.

† Assuming that 27% of the protein is still nonspecifically adsorbed, this percentage is based on the remaining 73%.

‡ This figure is *minimized* by the assumption that 27% of the refolded anti-BSA is nonspecifically adsorbed. If 12% is assumed nonspecifically adsorbed for both native and refolded fragment I, the calculated per cent renaturation would be 81%.

results nevertheless indicate again that about 65–80 per cent of the refolded protein retains its ability to combine with the conjugated antigen.

Discussion.—The experiments reported in this paper show that an active fragment of a rabbit antibody can be fully unfolded in concentrated guanidine hydrochloride. Several different measurements indicate that all noncovalent structure is destroyed under the conditions we have used. Upon removal of the guanidine, the protein recovers its original physical properties, and about 75 per cent of its ability to combine specifically with antigen. These data provide very strong evidence that different antibody specificities cannot be generated by different arrangements of noncovalent bonds in molecules of identical covalent structure. If noncovalent bonds represented the chemical basis for the biological specificity of antibodies, this specificity would be destroyed upon disruption of the noncovalent structure. In the test tube and in the absence of antigen, no appreciable number of molecules would then be expected to return to the conformation required to regenerate a given specific antibody site.

Recent data of Fleischman *et al.*[19] on the location of the disulfide bonds in γ-globulin also appear to eliminate the possibility that different antibody specificities can be generated (in large numbers) by different arrangements of disulfide bonds alone. According to their results, a completely reduced sample of fragment I would contain twelve sulfhydryl groups, not counting the SH group derived from the single highly labile bond[20] between the two active fragments of the parent molecules. These SH groups could combine to form $12!/6!2^6 = 10{,}395$ distinguishable patterns of disulfide bonds.[21] Included in this number would be arrangements with one, two, and three disulfide bonds linking the two polypeptide chains[19, 22] of fragment I to each other. Fleischman *et al.*[19] have found however that only one interchain bond is present, and that the remaining disulfide bonds are intrachain, three within the B chain, and two within that part of the A chain which remains in fragment I after papain cleavage. The number of distinguishable patterns which can be formed within the restrictions of such an arrangement is considerably less. If any one of the SH groups of the B chain and any one of the SH groups of the A chain fragment can combine to form the single interchain bond, then there are 35 ways of forming the interchain bond, and the total number of distinguishable arrangements becomes $(35)(6!/3!2^3)(4!/2!2^2) = 1{,}575$. It is, however, difficult to envisage restrictions which require the existence of a single interchain bond, but permit free choice among the SH groups which might form such a bond. If only a single such bond can be formed, it is likely that a specific pair of SH groups must be involved. This reduces the number of possibilities by a factor of 35, leaving only 45 possible patterns of disulfide bonds for a molecule with given amino acid sequence.

It has been suggested by Porter[22] and by recent data of Karush[23] that the active site of an antibody molecule resides entirely on the A chain. Since the active site unquestionably lies entirely on fragment I, the over-all conclusion would be that antibody specificity resides on the fragment of A chain which is part of fragment I. This fragment contains only two disulfide bonds, reducing the number of possible patterns to three.

One must conclude from these considerations that antibody specificity is generated primarily by differences in amino acid sequence in some portion of the antibody molecule. If this is true, then it should be possible to recover antibody specificity

from a molecule which has had not only its noncovalent interactions, but also its disulfide bonds, disrupted. However, initial attempts to carry out renaturation after disulfide bond reduction have not, so far, led to success. Further attempts are in progress.

The suggestion that differences in antibody specificity reflect differences in amino acid sequence is compatible with the finding of differences in over-all amino acid composition between different antibodies.[24] Such differences by themselves are not conclusive, however, since they could arise in other ways. A striking aspect of the published amino acid compositions is in fact the smallness of the differences. It suggests that the differences in amino acid sequence which are associated with antibody specificity may be confined to a small region of the antibody molecule.

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THE BIOLOGICAL STATUS OF LAMBDA AND RELATED PARTICLES IN PARAMECIUM AURELIA

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The biological status of kappa and related particles has long been the subject of intensive investigations and many speculations.[1] Kappa has been variously looked upon as an endogenous genetic element, a virus, a rickettsia, a bacterium, or a degenerate alga. The evidence in support of the various viewpoints has been scant and mostly of a circumstantial nature. Sonneborn[2] has called these particles infectious, intracellular parasites, manifesting a protocellular level of organization, which would set them apart from typical bacteria, being either degenerate bacteria or intermediates between viruses and typical bacteria. Their size, sensitivity to antibiotics, reproduction by transverse division, and the presence of both DNA and RNA in the same particle would make them resemble rickettsiae or bacteria. On the other hand, the reported lack or paucity of enzymes and the failure to grow extracellularly would make them resemble viruses. The concentrations of DNA and RNA in the kappa[3] and lambda particles[4] are very similar and do not distinguish these particles from bacteria.

We wish to report here the successful *in vitro* cultivation of the lambda particle together with some of its biological properties.

Materials and Methods.—Axenically grown lambda-bearing *Paramecium aurelia*, stock 299, were obtained from Dr. A. T. Soldo (Biology Division, Schering Corporation, Bloomfield, New Jersey) and were grown in the medium described by him.[5] They were periodically checked for the presence of lambda particles using the aceto-orcein stain. A culture of the lambda-bearing paramecia (150 ml containing 10,000 organisms per ml) was concentrated by centrifugation at 1000 × *g*, the supernatant was decanted and the organisms in the remaining 5 ml were homogenized according to the method of Sonneborn.[6] The resulting homogenate was inoculated into 5 ml of various media contained in 15 × 200 mm culture tubes. All operations were carried out under the strictest aseptic conditions, and the inoculated tubes were always checked for foreign bacterial contamination.

One of the components of the media was a yeast fraction prepared as follows (Dr. A. T. Soldo, personal communication): The nondialyzable fraction from baker's yeast (strain 1821, Standard Brands, Inc.)[7] was prepared by stirring baker's dry yeast into glass-distilled water at 60°(1:4, w/v). The mixture was stirred for 2 hr at 60°. After autoclaving at 120° for 20 min the mixture was centrifuged and the clear supernatant poured into a section of cellulose dialysis tubing, immersed in distilled water with the open end above the water. After autoclaving, the open end was tied off and dialysis continued in running distilled water for 2 days. The impermeate was dried in the frozen state. The resulting dry powder was refluxed with methanol (1:100, w/v) for 30 min. The mixture was filtered and the extraction repeated once. The powder was washed once with acetone. This powder was slurried into water (50 mg per ml) and centrifuged at 25,000 × *g* for 15 min. The sediment was dried *in vacuo*. According to Allfrey and King,[8] folic acid should have been removed completely from this preparation. It contains carbohydrate and protein but no nucleic acid or lipids. The treatment precludes the presence of nondenatured proteins or small peptides.

Tests for killing ability were carried out using *P. aurelia* of stock 92, syngen 3, grown in a monoxenic medium (baked lettuce infusion inoculated with *Aerobacter aerogenes*) as testers. Two hundred to 300 sensitive organisms, contained in a volume of approximately 0.1 ml, were placed

in wells of a depression slide and serial dilutions of the lambda particles were added. The depressions were observed for 90 min and the number of killed and lysed sensitives was recorded.

Results.—Of the various media tried, originally only the medium reported in Table 1 would support the growth of the lambda particles. The particles multiply at the rate of about one fission per day. Cultures of the lambda particles have now been maintained for 24 weeks, with transfers made every week. It has since been

TABLE 1

COMPOSITION OF THE MEDIUM FOR THE GROWTH OF THE LAMBDA PARTICLES

	Conc.*		Conc.
Glycine	40	Na EDTA	500
L-Alanine	60	Adenosine	500
DL-Serine	60	Edamine S	5 mg
DL-Threonine	70	Glycerol	10 mg
DL-Valine	40	Maltose	2.5 mg
DL-Leucine	50		
DL-Isoleucine	50		
DL-Methionine	60	Ca Pantothenate	5
DL-Asparagine	36	Nicotine amide	5
L-Glutamic acid	80	Pyridoxal H Cl	5
L-Aspartic acid	20	Pyridoxamine H Cl	2.5
L-Lysine H Cl	50	Riboflavin	5
L-Arginine H Cl	60	Folic acid	2.5
DL-Histidine H Cl	40	Thiamine H Cl	15
L-Phenylalanine	40	Biotin	1.25 m μg
L-Tyrosine	15	α-Lipoic acid	0.05
L-Tryptophan	30	Stigmasterol	0.4
L-Proline	40	Yeast fraction	500
$MgSO_4$	250	0.001 *M* Na phosphate pH 6.8	20 ml/100 ml
Guanosine-mono-phosphate	250		
Uridine-mono-phosphate	250		

* All concentrations are in μg/ml, unless otherwise specified.

found that exogenous folic acid is not required, thereby confirming the observation of Soldo[9] that the lambda particles can synthesize folic acid.

The observation of Soldo that lambda particles are gram-negative rod or diplorod type structures[9] was confirmed with the *in vitro* grown particles (Fig. 1). The particles have retained their ability to kill sensitive paramecia, as can be seen from Table 2. There is a fair degree of proportionality between the number of particles present and the number of sensitives killed. If axenically grown sensitives are used, the killing action of comparable numbers of lambda particles is about 60 per cent less.

TABLE 2

THE KILLING ACTION BY INCREASING AMOUNTS OF LAMBDA PARTICLES

No. of λ particles	No. sensitives killed
6	0
30	3
60	9
120	21

Lambda particles grown *in vitro* were tested for their ability to infect cells of stock 299, which had become sensitives through the loss of the particles. An axenic stock of 299-sensitive cells was cultured in a medium containing 100 units of penicillin per ml for 4 days in order to ensure that the animals were lambda-free.[9] They were then transferred to the regular axenic medium. One hundred to 200 sensitive paramecia contained in 0.2 ml of culture medium were placed in the well of a sterile depression slide. To this were added 0.02 ml of a suspension of lambda particles containing approximately 400 particles. The depression slides were placed in a

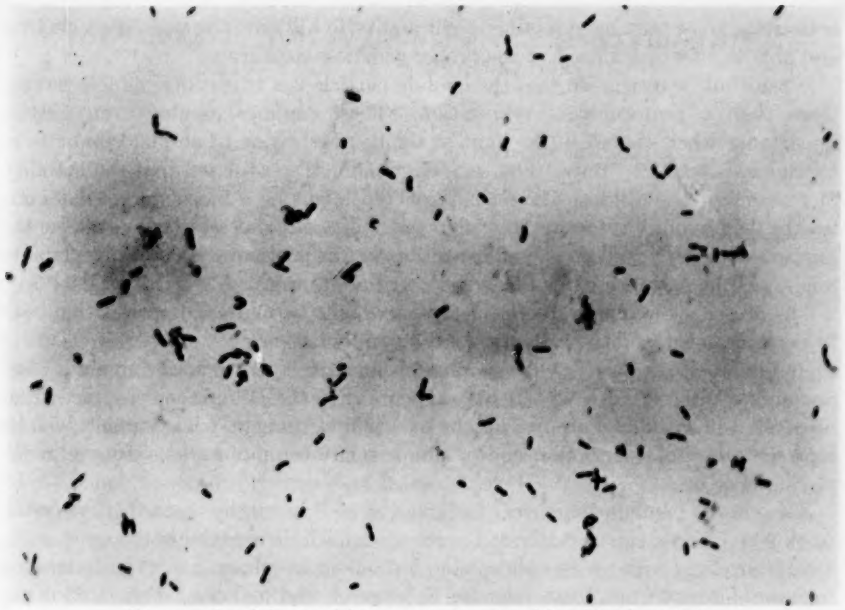

FIG. 1.—Lambda particles grown *in vitro*. Magnification ×715.

moist chamber and left overnight. The next day the surviving animals (about 50% of the original) were transferred to tubes containing the axenic medium and allowed to grow up. At the end of 5 days these animals were transferred to a lettuce infusion inoculated with *A. aerogenes*. In this bacterized state the animals were tested for their killing ability in the usual manner. Blistering and lysis of sensitive animals were observed within 30 min. The infected animals were also stained with aceto-orcein, and the presence of the lambda particles in the cytoplasm was ascertained.

Discussion.—The above findings necessitate a re-evaluation of the biological status of kappa and its related particles. These particles are clearly endosymbiotes, which can also be grown *in vitro*. Various reports in the literature emphasize the postulated fact that these particles demonstrate a lack, or at least a paucity, of enzymes. This assumption is invalid. First, Soldo[9] has demonstrated that the presence of the lambda particles eliminates the requirement of folic acid for stock 299 of *P. aurelia*. Since the lambda particle can also grow in an axenic medium in the absence of folic acid, it therefore possesses the elaborate enzymatic machinery for the synthesis of folic acid. Second, the ability of the lambda particle to grow *in vitro* indicates that the particle must be able to synthesize its cellular components from the constituents of the medium. The lambda particle requires, as does *P. aurelia*, a factor (or factors) present in the yeast fraction. Thus far, attempts to replace this fraction with known substances have been unsuccessful. The original homogenate is diluted out in serial transfer and thus is not contributing other unknown nutrilites to the medium.

The *in vitro* culture of the lambda particles has not changed their biological char-

acteristics, since they have retained their ability to kill sensitive paramecia and are also able to infect paramecia of the proper genetic constitution.

It can thus be concluded that the lambda particle is a true endosymbiote having more than a protocellular organization. These findings assume even greater importance when viewed in the light of the demonstration of an endosymbiote in *Crithidia oncopelti*.[10] Butzel and van Wagtendonk[11] postulated that the inability of *P. aurelia* to synthesize the purine skeleton might be a biochemical characteristic of the phylum Protozoa. The reported ability of *C. oncopelti* to synthesize the purine skeleton[12] would not be an exception to this postulate. The endosymbiote might well be responsible for the synthesis of the purines.

The presence of endosymbiotes in protists might be more widespread than was hitherto assumed, and thus account for some of the atypical growth requirements of some of the representatives of this phylum and their ability to accumulate large amounts of nucleic acid.[13,14] It also appears that the cytoplasmic particles first discovered in *P. aurelia* are not unique as was first thought. They might well be representatives of a large host-endosymbiote relationship of various degrees in the phylum Protozoa.

Summary.—Lambda particles, obtained from axenically grown *P. aurelia*, stock 299 (killers), can be cultivated *in vitro* in a medium containing 17 amino acids, 8 vitamins, guanosine-mono-phosphate, uridine-mono-phosphate, stigmasterol, a fraction obtained from yeast, edamine S, glycerol, and maltose. Folic acid is not required. The *in vitro* grown particles have retained their killing ability and can, under appropriate conditions, infect sensitive *P. aurelia* of stock 299. It is concluded that these particles are bacterial endosymbiotes of *P. aurelia*, stock 299.

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*SYNERGISTIC ACTION OF NITROGEN MUSTARD AND RADIATION IN MICROORGANISMS**

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It is likely that the production of structural defects in DNA is a prominent molecular event relevant to cellular inactivation by such diverse agents as X rays, ultraviolet light (UV), and the radiomimetic alkylating agent, nitrogen mustard.[1–3] Although the chemical nature of these defects and the reaction mechanisms involved in their formation are undoubtedly different for each agent, the existence of an important cellular target makes a comparison of their respective inactivation kinetics especially interesting,[4] and also raises the possibility that synergistic interactions[5] might occur among them.[6]

Recent studies have indicated that certain chemical modifications of the structure of DNA, such as the incorporation of halogenated base analogues, result in an increased cellular response to both X rays and ultraviolet light.[7–10] Furthermore, bacteria containing sublethal UV photoproducts, a large fraction of which are photoreactivable and presumably in DNA, have an increased sensitivity to X rays.[11, 12] Since it also has been shown that nitrogen mustard (HN2) can produce interstrand cross links and chain breaks in DNA *in vitro*,[13, 14] one might reasonably expect that any such structural defects produced sublethally *in vivo* could also serve to enhance the killing effect of ultraviolet and ionizing radiation.

First, this paper describes our technique for obtaining reproducible HN2 dose-effect curves for bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*) as a function of the initial HN2 concentration in the cell suspensions. For haploid and diploid yeast the resulting HN2 curves are similar in shape to the corresponding X ray survival curves, that is, exponential for haploid and sigmoid for diploid.[6] Second, interaction experiments have shown that HN2 and 2537 A ultraviolet light act synergistically in the inactivation of haploid and diploid yeast, and *E. coli* B/r. On the other hand, while HN2 and X rays interact synergistically in B/r and diploid yeast, no such interaction occurs in haploid yeast.

Materials and Methods.—Cultures of haploid and diploid yeast (*Saccharomyces cerevisiae*, SC-7 and SC-6) were grown for 6–8 days on potato-dextrose-agar (Difco) slants; *E. coli* B and B/r were grown for 18–20 hr either in nutrient broth or 1% peptone. The cells were then harvested, washed, and suspended in *M*/15 phosphate buffer (pH = 7.0) prior to treatment with 150 kv X rays (HVL = 1 mm Al), 2537 A ultraviolet light, or methyl bis (β chloroethyl) amine hydrochloride (HN2). The suspensions were aerated by bubbling with sterile, water-saturated air during all X irradiations in order to maintain a maximum radiobiological oxygen effect. Yeast survivors were scored by their ability to form visible colonies on potato-dextrose-agar plates following 4 days' incubation at 28°C; the *coli* were plated on nutrient (salt) agar, and visible colonies counted after 18 hr incubation at 37°C. Irradiation and calibration techniques for the X ray and UV sources have already been described;[15] the X ray dose rate was 4.37 kilorads/min, and the incident UV flux (90% at 2537 A) was 20 ergs/mm^2/sec.

Inactivation by HN2 was effected in the following manner: a concentrated aqueous solution of nitrogen mustard was made by dissolving a known amount of the desiccated hydrochloride salt in a known volume of the phosphate buffer. One ml of this solution was then diluted with 9 ml of cell suspension within 20 sec after preparation, producing the initial "exposure" concentration. The suspensions were agitated continuously at room temperature (24°C), and samples were

removed and plated at various times following the addition of the chemical. The fall in viable titer of *E. coli* B for several initial HN2 concentrations is shown in Figure 1. After approximately 3 hr incubation under these conditions (6 hr for yeast), there is no further change in viable titer, although in some instances a partial recovery occurs if the incubation time is extended to 1 day or more.[16] However, a definable plateau exists for several hours, and this was used as the biological end point in these experiments. The inactivation kinetics are the result of several complex processes, such as cyclization and hydrolysis of the mustard to less active forms, its diffusion across cell walls, and its fixation on cellular sites. Since the plateau in viable titer is determined by the amount of HN2 added to the suspensions, it is possible to construct "dose-effect" curves by plotting the plateau survival as a function of the initial HN2 concentration (Figs. 2 and 3).

Results. —(1) *Nitrogen mustard dose-effect curves for E. coli and yeast:* The plateau survival for *E. coli* B and B/r as a function of initial HN2 concentration is shown in Figure 2. It is seen that B/r is more resistant to HN2 inactivation than B. These results are similar to those of Bryson,[17] except that under our experimental conditions both B and B/r appear to be significantly more sensitive to HN2 inactivation. This apparent difference in HN2 sensitivity may be due to the fact that Bryson incubated the cells with HN2 for only 1 hr before plating.

Nitrogen mustard dose-effect curves for haploid and diploid yeast are shown in Figure 3. These curves are strikingly similar in shape to the corresponding X ray survival curves for these cells, although the LD90 dose ratio between the two ploidies is somewhat less, being about 4 for HN2 as opposed to 5 for X rays. (Since it is unlikely that this reflects any similarity in the chemical actions of these agents on DNA or other cellular components, it is apparent that one cannot rigorously infer details of the initial physicochemical events involved in cellular inactivation solely from the intrinsic shapes of dose-effect curves. However, it might be argued that once the relevant macromolecular defects are established by the direct chemi-

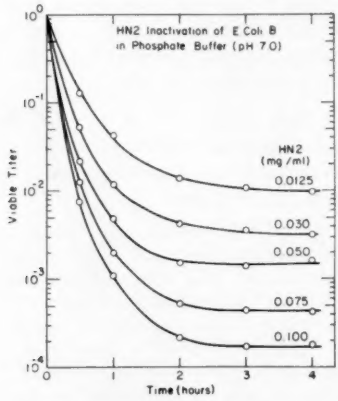


FIG. 1.—Viable titer of a stationary suspension of *E. coli* B in *M*/15 phosphate buffer (pH = 7.0) as a function of time after the addition of various initial amounts of the HN2.

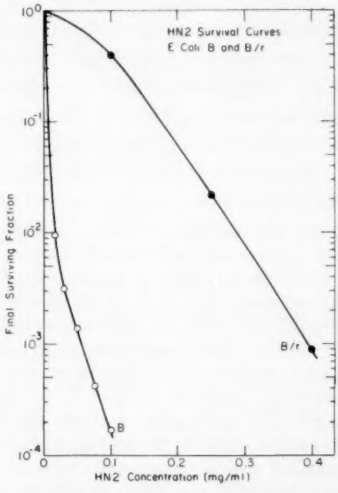


FIG. 2.—HN2 dose-effect curves for stationary phase *E. coli* B and B/r. Abscissa: initial HN2 concentration in the suspensions; ordinate: final surviving fraction (Fig. 1) reached after several hours' incubation at 24°C.

cal actions of such agents, then similarities in the shape of survival curves probably reflect similarities in the subsequent biochemical failure which takes place upon stimulation of cell division and which is the more immediate cause of reproductive death.)

(2) *Interactions of nitrogen mustard with X rays and UV:* In these experiments, suspensions of interdivisional yeast or stationary phase *E. coli* B/r were treated first with HN2, so as to reach a given plateau survival level, and then further inactivated with graded doses of either X rays or 2537 A ultraviolet light. In this way, one can study the combined effect of sublethal HN2 damage and radiation. It should be noted that, under the conditions of the experiments, the corresponding X ray and UV survival curves for both haploid yeast and *E. coli* B/r are similar in shape, i.e., exponential for X rays and sigmoid for UV.

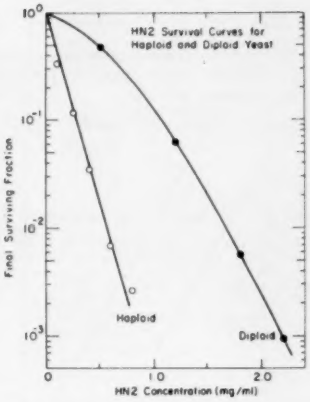


FIG. 3.—HN2 dose-effect curves for suspensions of interdivisional haploid and diploid yeast.

(a) *Haploid yeast:* The upper curve in Figure 4 is a typical exponential X ray survival curve for interdivisional haploid yeast; below it is the X ray survival curve of another sample of the same suspension whose viable titer was reduced to 5 per cent by preliminary treatment with nitrogen mustard. Since these curves are parallel to one another, it is clear that HN2 treatment does not affect the X ray sensitivity of these cells. Similarly, if the order in which the two inactivating agents are applied to the cells is reversed, it is found that preliminary X ray exposure has no effect on the subsequent HN2 dose-effect curve. Thus, it would appear that there is no interaction between HN2 and X rays for the inactivation of interdivisional haploid yeast.

In contrast to this lack of interaction between HN2 and X rays, HN2 and 2537 A ultraviolet light act synergistically in the inactivation of these cells. The UV survival curve for interdivisional haploid yeast pretreated with HN2 is shown in Figure 5 (lower solid curve), where it is to be compared with that of another sample of the same population which was untreated with HN2 (upper solid curve). The broken curve labeled "no additivity" is simply the "UV only" curve displaced downward to the origin of the interaction (HN2 + UV) curve, while the curve labeled "complete additivity" is that portion of the "UV only" curve below the 2.5 per cent survival level (cf. ref. 6).

Thus, the synergism between HN2 and UV is manifested as a partial removal of the shoulder from the UV survival curve, with no apparent change in its limiting slope. The kinetics of the interaction have a rather more dramatic appearance when the order of the inactivating treatments is reversed: preliminary UV exposure which inactivates to the 10 per cent survival level serves to increase 2- to 3-fold the exponential slope of the HN2 dose-effect curve. These latter experiments are still in progress and will be described in more detail in a future paper.[18] It is sufficient here merely to note that the interaction between HN2 and UV in haploid

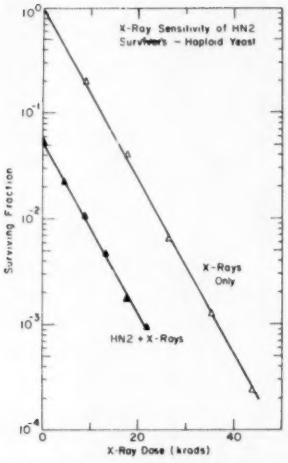


FIG. 4.—Demonstration of zero interaction between HN2 and X rays in haploid yeast.

yeast is synergistic for both orders in which the two inactivating agents may be applied to the cells.

(b) *Diploid yeast:* The interaction between HN2 and UV in interdivisional diploid yeast is synergistic, and the kinetics are essentially similar to those shown in Figure 5 for haploid yeast. However, it is noteworthy that, unlike haploid yeast, HN2 and X rays also interact synergistically in diploid yeast: HN2 pretreatment serves to remove the shoulder partially, but does not affect the limiting slope of the X ray survival curve (Fig. 6). The fact that neither HN2 nor UV[6] interact with X rays in the inactivation of haploid yeast, whereas both interactions are synergistic in diploid yeast, again serves to emphasize the importance of physiological events in the cell in determining the shapes of survival curves following treatment with X rays, UV, and/or HN2 (this point is further elaborated below).

(c) *Escherichia coli B/r:* The interaction between HN2 and UV in *E. coli* B/r is similar to that for haploid and diploid yeast, although it would appear that the slope of the "HN2 + UV" curve does not attain parallelism with the "UV only" curve in the dose region investigated (Fig. 7). However, it is clear that the interaction is synergistic, and HN2 pretreatment leads to a partial removal of the shoulder from the UV survival curve. In the reverse order, it has been found that preliminary UV exposure serves to remove the shoulder of the subsequent HN2 dose-effect curve.[18]

Again in contrast to the case of haploid yeast, HN2 pretreatment increases the X ray sensitivity of *E. coli* B/r (Fig. 8). The broken line in Figure 8 is drawn parallel to the "X ray only" curve to show that the interaction curve (HN2 + X rays) has a somewhat steeper slope (dose-modifying factor = 1.25). Thus, although both haploid yeast and *E. coli* B/r have exponential X ray survival curves, HN2-treated B/r are sensitized to X rays, whereas haploid yeast are not; but their UV responses after HN2 treatment are similar.

Discussion.—The results of the various interaction experiments among X rays (delivered under fully oxygenated conditions), 2537 A ultraviolet light and nitrogen mustard in haploid and diploid yeast, and *E. coli* B/r are summarized in Table 1. The UV-X-ray interaction results have been reported elsewhere[6, 11, 12] and are included here for convenience in this discussion. It should be noted that there is no interaction (denoted by "0" in the table) between X rays and either HN2 or UV in haploid yeast, whereas all possible interactions among these agents are synergistic in both diploid yeast and *E. coli* B/r (denoted by "+" in the table). Thus, it seems reasonable to regard haploid yeast as the anomalous organism in terms of these experiments, and to inquire if this pattern of interactions can be related to any other contrasting properties of haploid and diploid yeast. However, we must first consider the significance of the interactions in *E. coli* B/r, the existence of which is consistent with the view that the killing effect of ionizing and ultraviolet radiations is enhanced in cells whose DNA contains a sufficient number of sublethal

structural alterations or defects. (Similarly, radiosensitization by incorporated DNA base analogues[7-10] and the X ray sensitizing effect of UV photoproducts[11, 12] are consistent with this general hypothesis.)

TABLE 1

INTERACTIONS AMONG VARIOUS INACTIVATING AGENTS IN YEAST AND *E. coli*

Agents combined†	Nature of Interaction* *S. cerevisiae* Haploid	*S. cerevisiae* Diploid	*E. coli* B/r
HN2, X rays	0	+	+
HN2, UV	+	+	+
UV, X rays‡	0	+	+

* Synergistic interactions are denoted by "+," no interaction by "0."

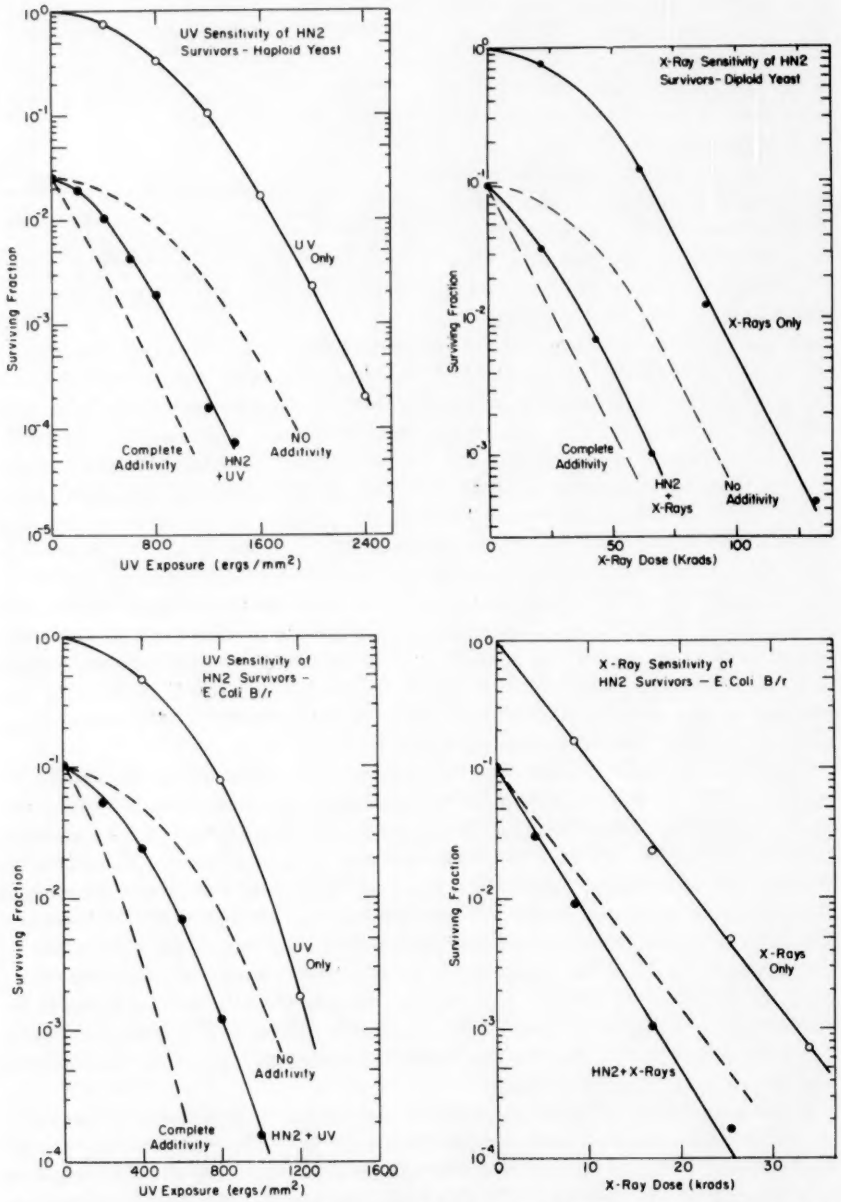
† In all cases the order in which the agents are applied to the cells can be reversed without affecting the qualitative nature of the interaction; for example, the interaction between HN2 and X rays is zero in haploid yeast and synergistic in B/r independently of whether the HN2 treatment is given before or after the X rays.

‡ See ref. 6 for haploid yeast, refs. 11 and 12 for B/r.

The most straightforward interpretation would be that interactions arise directly from reactions among the various products of chemical and radiation attack on cells.[19] However, it has recently been suggested that bacteria may possess enzymatic reactivation mechanisms which can either repair or effect a bypass of radiation damage in DNA.[20-25] Thus, it is interesting to speculate on the possibility that synergistic interactions can be regarded operationally as arising from the inhibition of such reactivation processes. For example, independently of the precise mechanisms of the reactivation and their inhibition, one might assume that the probability of repair or bypass of any individual, but potentially lethal, DNA structural defect is reduced by the presence of any other neighboring defect within some arbitrary distance along the polynucleotide chain. This assumption implies that the dose-effect curves for agents which inactivate by attack on DNA should possess a shoulder, except in cases where the reactivation mechanisms are extremely inefficient or nonexistent, or the defects produced up to any practical dose level are so few and sparsely distributed along the polynucleotide chain as to be effectively independent of one another (e.g., no more than 1 in 25, and perhaps as few as 1 in 200, cistrons would be intersected by 100-ev ion clusters after a D_{37} X ray dose of 6.3 krads to *E. coli* B/r).

It will be recalled[20-24] that the UV sensitivity of bacteriophage T1 can be increased, either by UV irradiation of the host cells, by substituting 5-bromouracil for thymine in the phage DNA, or by plating the irradiated phage on the radiation-sensitive mutant *E. coli* $B_s{-}1$.[26, 27] The first two of these effects presumably arise through the inhibition of reactivation processes in the host cells, while the mutant $B_s{-}1$ evidently lacks one or more of the reactivation enzymes.[23, 28] At least two general reactivation mechanisms may be involved: (*a*) functional elimination of the damaged region of the phage DNA by genetic exchange with homologous regions of the bacterial DNA;[20] and/or (*b*) restoration of the damaged region by direct enzymatic repair or local DNA synthesis.[25] These possibilities need not be mutually exclusive,[29] in that the reactivation enzymes may also serve some normal function in recombination processes.

If the interactions in yeast are similarly regarded as arising from the inhibition of reactivation processes, then the results shown in Table 1 would suggest that haploid yeast is unique in that it would appear to be incapable of either repairing or bypassing X ray damage. On this basis, the peculiar pattern of interactions in haploid as opposed to diploid yeast parallels the situation with regard to recovery of viability as observed upon delayed plating after treatment with either X rays, UV, or HN2: diploid yeast is capable of recovery after treatment with any one of these agents; but whereas haploid yeast is capable of some recovery after UV or



FIGS. 5–8.—Synergistic interactions between HN2 and UV or X rays in haploid and diploid yeast, and *E. coli* B/r.

HN2 treatment—hence, perhaps, the observed HN2-UV interaction—it is totally incapable of recovery after X irradiation.[16] Thus, it may be more than coincidental

that the only two cases of zero interaction involve haploid yeast and X rays, and that the only instance of no recovery upon delayed plating is for haploid yeast after X irradiation.

While it is premature to speculate on the reasons for the apparent absence of reactivation or recovery processes relevant to X ray damage in haploid yeast, several interesting possibilities come to mind: (1) haploid cells may be able to elaborate reactivation enzymes, but the X ray damage most relevant to inactivation may not be located in genetically functional DNA, but rather in some other, nonrepairable, structure; (2) with its strong tendency toward respiratory deficiency (absence or failure of aerobic metabolism), and since respiratory deficient diploid cells are incapable of recovery,[16] haploid yeast may be unable to produce enough ATP to drive the reactivation processes; (3) repair of X ray damage may be effected in diploid yeast only by recombination events, either during DNA replication or chromosomal pairing, which do not occur in the haploid strains. Experiments designed to test these various hypotheses are now in progress.[30]

Summary.—(1) A technique is described for obtaining reproducible "dose-effect" curves (analogous to radiobiological survival curves) for the inactivation of microorganisms by the bifunctional alkylating agent, nitrogen mustard. (2) The nitrogen mustard dose-effect curves for haploid and diploid yeast are similar in shape to the corresponding X ray survival curves for these organisms, i.e., exponential for haploid and sigmoid for diploid The LD90 dose ratio between the two ploidies is 4 for HN2 and 5 for X rays. (3) *E. coli* B/r is more resistant to inactivation by HN2 than is *E. coli* B. (4) Nitrogen mustard acts synergistically with both X rays and ultraviolet light in the inactivation of *E. coli* B/r, and diploid yeast, but only with UV in haploid yeast. (5) It is suggested that the formation by HN2 of sublethal structural defects in DNA serves to enhance the killing effect of both ultraviolet and ionizing radiations, and that synergistic effects arise through the mutual inhibition of reactivation processes which would otherwise serve to repair or bypass some fraction of the molecular damage.

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Abbreviations used in this paper: DNA, deoxyribonucleic acid; UV, ultraviolet light; HN2, nitrogen mustard, methyl bis (β chloroethyl) amine hydrochloride; ATP, adenosine triphosphate.

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magnitude of the biological effect can be expressed in terms of the surviving fraction of cells, an interaction is described as synergistic, zero, or antagonistic, according as the net effect of the two agents together is greater than, equal to, or less than the sum of the logarithms of their independent effects (i.e., the product of the two independent surviving fractions for each agent alone). For synergistic interactions, the effect of the first agent on the survival curve of the second can be to remove an initial shoulder, increase the limiting slope, or both; the effect is properly described as a sensitization *only* if the limiting slope is increased.

Other types of synergistic effects among HN_2, UV, and X rays have also been reported. For example, in *Aspergillus terreus* and *Neurospora crassa*, the UV-induced mutation rate is significantly enhanced by pretreatment with amounts of HN_2 which, when used alone, have no appreciable mutagenic effect. Also, in *Aspergillus*, the mutation rate for combined UV and X ray treatments is enhanced beyond that expected on the basis of simple additivity of the effects [Swanson, C. P., W. D. McElroy, and H. Miller, these PROCEEDINGS, **35**, 513 (1949); Swanson, C. P., *J. Cell. Comp. Physiol.*, **39** (Suppl. 1), 27 (1952)].

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AN ANALYSIS OF SV40-INDUCED TRANSFORMATION OF HAMSTER KIDNEY TISSUE IN VITRO, II. STUDIES OF THREE CLONES DERIVED FROM A CONTINUOUS LINE OF TRANSFORMED CELLS

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In an earlier report on the characteristics of transformation of hamster kidney cell cultures by SV40 virus, a continuous cell line (THK-1) of transformed cells was described.[1] This cell line showed two unusual characteristics, namely, the continued presence of SV40 in a form or amount difficult to detect, and induction of transplant tumors containing both carcinomatous and sarcomatous elements. The interpretation of both of these findings depends on whether the cells are homogeneous in these respects, or whether the findings are the result of mixtures of cell types which are heterogeneous with respect to carriage of virus and type of growth *in vivo*. To clarify these points, clones were established, in the presence of antiserum, from the THK-1 cell line, and cell lines established from them. In this report, a study of three such clones and the tumors produced in hamsters by transplantation of the cloned cells will be presented. These three clones have all been found to yield infectious virus when the clone cells are implanted on Cercopithecus kidney monolayer cultures, providing further evidence for the integration of the SV40 viral genome in a subinfectious state in transformed hamster kidney cells.

Materials and Methods.—Cloning technique: Cells were cloned from the continuous transformed line THK-1, without the benefit of feeder cells; the cloning procedure of Puck[2] employing small cylinders was employed. Cells of the 29th passage were dispersed by trypsinization; a sample was counted in a hemocytometer, and the cells were observed to be monodisperse. One hundred cells in 5 ml of growth medium were dispensed into each of four plastic Petri dishes 60 mm in diameter. The growth medium consisted of NCTC-109 with 15% unheated fetal calf serum, 0.5% heated hyperimmune rabbit anti-SV40 serum (neutralizing antibody titer > 2560), and antibiotics. The cultures were incubated at 37° C in 5% CO_2-humidified atmosphere. One dish was examined on the 3rd day and tiny clones containing 2–4 cells were present. The remaining cultures were left undisturbed until the 7th day. At this time there was an average of 35 colonies per plate. Three small, well-isolated colonies, two (#4-2 and #4-2) epithelioid and one (3-3) of more fibroblastic appearance, were considered to represent clones and were selected for passage. A sterile penicillin assay cylinder was rimmed with sterile silicone stopcock grease (Dow-Corning) and placed over each clone; the cells were removed by trypsinization and transferred to separate culture dishes. The cells grew rapidly and formed confluent monolayers in 6–10 days. The growth medium containing antibody was used for 6 passages; thereafter, the same medium was used, but without the rabbit antiserum.

Virus isolation from clone cells: The four methods used for determination of virus in transformed cultures have been described.[1] In the overlay method, the clonal lines so rapidly overgrew the monkey kidney (AGMK) cells that the latter cells could not be observed for more than 14–17 days in most instances. Therefore, blind passages were made, generally at the time of complete overgrowth, by disrupting cells by freeze-thawing the culture tubes 2–3 times or extracting with chloroform,[1] pooling the contents of replicate cultures, and inoculating 0.1 ml of the disrupted cell suspension into three AGMK culture tubes. These were observed for 30–40 days for occurrence of characteristic SV40 cytopathic effects (CPE).

Transplantation: Procedures used for transplantation of tissue culture cells into weanling hamsters and for viral and histologic studies have been described previously.[1, 3] Tumors which

appeared purely sarcomatous by histological examination were resectioned at four different levels of the tumor in order to verify the absence of epithelial elements.

Chromosome studies: Cytogenetic studies were performed by techniques previously described.[4]

Results.—Growth characteristics in tissue culture: The three clonal lines have been carried without difficulty through 40 serial passages in tissue culture over a period of 196 days after cloning. The cells grow very rapidly and produce marked acidification of the medium. Through most of the passage series, clone 3-3 was composed of a spectrum of cell types ranging from thin fibroblastic cells to short triangular-shaped fibroblasts with a few cells of epithelioid appearance. Few giant cells were present. The cells grew in strands and whorls in a random, crisscross fashion and piled up to form nodular growth centers (Fig. 1). These cells loosened individually when exposed to trypsin. At the 37th passage, stained preparations showed predominantly polygonal cells. Clones 4-1 and 4-2 are composed of polygonal, epithelioid cells which grow into thick, multilayered sheets. Many more giant cells are present than in clone 3-3 (Fig. 1). When treated with trypsin, these clones loosen as sheets of cells. Some cells from all clones contain eosinophilic cytoplasmic masses of various sizes; similar structures have been seen in uninoculated primary cultures of weanling hamster kidney cells.

Isolation of virus from clone cells: The virus recovery data on the three clonal lines are summarized in Table 1. No virus was detected in the supernatant culture fluids at any time. With one exception, extracts of concentrated cell suspensions containing 2–4 $\times$ 10^6 cells/ml were free of detectable virus; an extract of 6th-passage clone 3-3 cells contained a trace amount of virus. However, virus was readily recovered from all three clonal lines by the overlay procedure (Table 1); in almost all instances blind passage was necessary for virus detection. Control AGMK cultures, extracted and passed in a similar fashion with each overlay test, have never yielded SV40.

Transplantation of clone cells to hamsters: 2–3 $\times$ 10^6 cells of each clone at the 5th passage level were inoculated subcutaneously into seven weanling hamsters. Tiny subcutaneous nodules developed at the site of inoculation in all animals at 4–6 weeks and grew to a size of 1 cm (mean tumor diameter) within 2–3 weeks. Tumors arising from the cells from clone 3-3 grew much faster than those arising from the other two clones. Five tumors from clone 3-3 and four each from clones 4-1 and 4-2 were examined histologically and tested for virus content. SV40 was recovered from two of the 3-3 tumors, but no virus was detected in extracts of the 4-1 and 4-2 tumors (Table 1). It is possible that these tumors would have yielded virus if tested by the overlay method.[5, 6]

Grossly, the tumors resembled those arising from transplantation of THK-1 cells.[1] Often the entire central portion of the tumor was necrotic. The tumors remained localized and metastases were never observed grossly in six tumorous animals. Histologically, the tumors from the cells from clone 3-3 were all sarcomas in varying stages of malignancy, closely resembling primary tumors in virus-inoculated hamsters as well as the purely sarcomatous tumors often produced by transplants of the parent THK-1 cell line. (See Fig. 2(*B*) in ref. 1.) Several tumors had areas which were very cellular and anaplastic as well as areas of frank fibrosarcoma. Some tumors arising from cells of clones 4-1 and 4-2 were purely sarcomatous, but three tumors from clone 4-1 and one from clone 4-2 had many areas

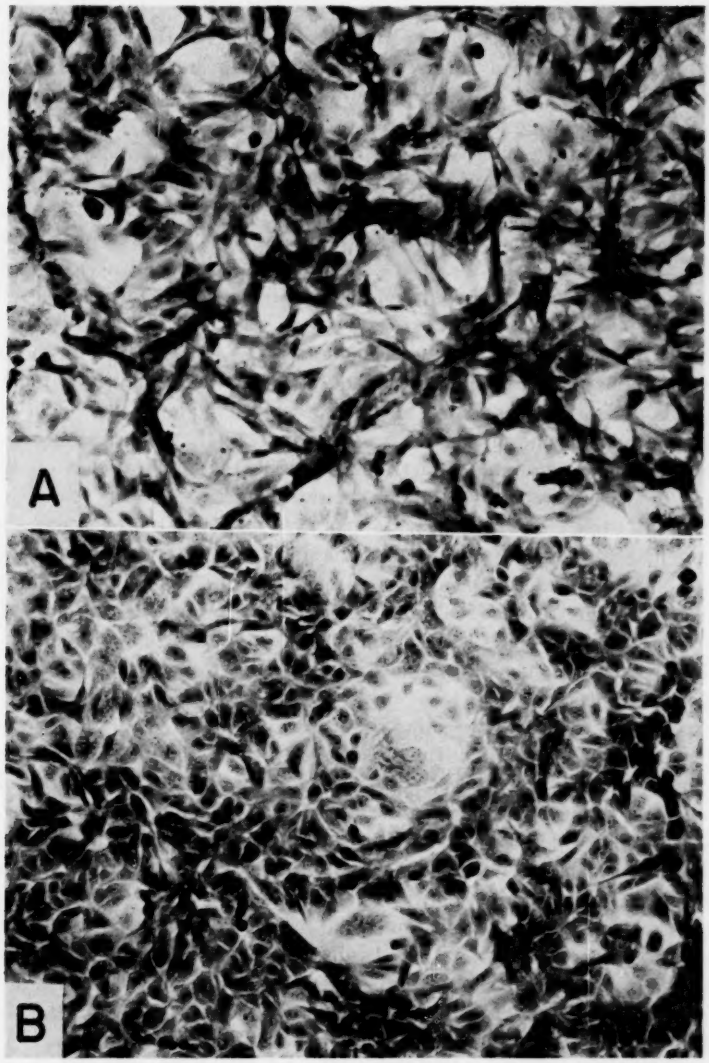

FIG. 1.—(*A*) Clone 3-3, 9th passage. Note cellular pleomorphism but predominantly fibroblastic morphology. H & E, ×143. (*B*) Clone 4-2, 9th passage. Note epithelioid morphology of cells, the multinucleated giant cells, and the numerous mitotic figures. H & E, ×143.

of epithelial differentiation into tubular structures, as well as sarcomatous areas (Fig. 2). These mixed tumors were identical to the carcinosarcomas frequently produced by the THK-1 cells. (See Fig. 2(*A*) in ref. 1.) All tumors from the clone cells had many multinucleated giant cells. Some tumors of both mixed and sarcomatous histology had invaded adjacent muscle; several blood vessels in both types of tumor also contained tumor cells within their lumen.

Chromosome studies: Prior to cloning, the THK-1 cell line showed marked variability in chromosomal number and morphology. A distribution analysis of the chromosome numbers of the clones was made at the 3rd, 10th, and 35th passage levels (Fig. 3). At the third passage after cloning, each of the three clones showed a different predominating modal chromosome number.

At the tenth passage, clone 3-3 still showed a marked predominance of the same modal chromosome number, with a narrow distribution about the mode. Clone 4-2 also showed its original modal number, but the distribution about the mode had a greater spread than in the earlier passage, and the frequency of cells with the modal number was lower than at the third passage. Clone 4-1 also showed an increase in the width of the distribution about the mode and a considerable reduction in the frequency of modal cells.

At the 35th passage, all three clones showed marked variation in chromosome numbers. Clone 3-3 had a new modal number with relatively low frequency of modal cells. Clone 4-1 appeared to have become bimodal, neither of the modes having the same chromosome number as the mode of the earlier passages. Clone 4-2 had a chromosome-number distribution similar in shape to that of its earlier passages, but an entirely new modal number was present.

Thus, in all three clones, during serial passages there was a progressive alteration of the original stem-like karyotype (presumably that of the cell from which the clone derived). Detailed karyotype analyses are being carried out to determine whether there is any tendency toward stabilization of one or a few karyotypes or whether the clones will ultimately come to resemble the parent line. These studies will also determine whether the modal cells constitute a true stem line, or are actually karyotypically heterogenous.

Discussion: From the preceding experiments, it is clear that transformed hamster kidney cells, cloned in the presence of high-titered antiserum, yield infectious virus material. There seems to be no question that the clones could have been infected with extracellular virus at the time of cloning because the culture fluids had been devoid of infectious virus for 26 passages; in addition, the amount of antiserum used should have been far more than adequate to neutralize any trace amounts that may have been present. Although only three clones were studied (and on a purely statistical basis this would only give assurance (at the 0.05 level) that at least 37 per cent of the THK-1 cells would yield virus), it is quite probable that all the transformed cells have this potential. Thus, clones of both of the cell types present in the original culture gave comparable results. Also, the similarity of these results to those of Sabin and Koch[7] supports this contention; their investigations indicated that a high proportion of hamster tumor cells induced by SV40 *in vivo* contain subinfectious SV40 viral genome.

Although it is clear that the viral genome is present in a high proportion of the transformed cells, with the exception of one test (24th passage of THK-1[1]), they contained less than one $TCID_{50}$ of extractable virus per 10^6 cells. Whether the infectivity recovered by the overlay method represents whole virus or infectious DNA[8] being liberated by cells, either as a rare burst or as an extremely slow trickle from a high proportion of cells, is not known. While it is conceivable that the viral DNA is being replicated without concomitant replication of viral protein components, there is strong evidence that the viral genome is efficiently directing synthesis

TABLE 1

RECOVERY OF SV40 FROM THREE CLONAL LINES OF TRANSFORMED HAMSTER KIDNEY CELLS

Clone	Expt.	Passage	Test for virus in supernatant	Test for virus in disrupted cells	Overlay Method			Blind passage result	Virus in tumors
					No. cells overlaid (log_{10})	Result	Length of observation (days)		
3-3	1-8	5							2/5[d]
	1-15	6	−	+[a]					
	1-31	9			5.8	−	17	+	
		9			5.2	−	17	+	
		9			4.2	−	17	+	
	3-14	18			5.6	−	11	−	
		18			4.6	−	11	+	
	3-27	21	−	−	5.6	−	14	−	
		21			4.6	−	34		
	4-18	28		−					
	5-23	35			5.2	−	18	+	
					4.2	−	18	+	
					3.2	−	18	+	
	1-8	5							0/4
4-1	1-15	6	−	−					
	1-31	9			5.8	−	17	+	
		9			5.2	+(1/3)[b]	17	+[c]	
		9			4.2	−	17	+	
	3-14	18			5.6	−	11	+	
		18			4.6	−	11	+	
	3-27	21	−	−	5.6	−	14	+	
		21			4.6	+(1/3)	34		
	1-8	5							0/4
	1-15	6	−	−					
4-2	1-31	9			5.8	−	17	+	
		9			5.2	−	17	+	
		9			4.2	−	17	+	
	3-14	18			5.6	−	11	−	
		18			4.6	−	11	+	
	3-27	21	−	−	5.6	−	14	+	
		21			4.6	+(2/3)			

[a] Positive on blind passage.
[b] No. tubes positive/no. tubes inoculated.
[c] The two negative tubes were blind passed.
[d] No. tumors positive/no. tumors tested.

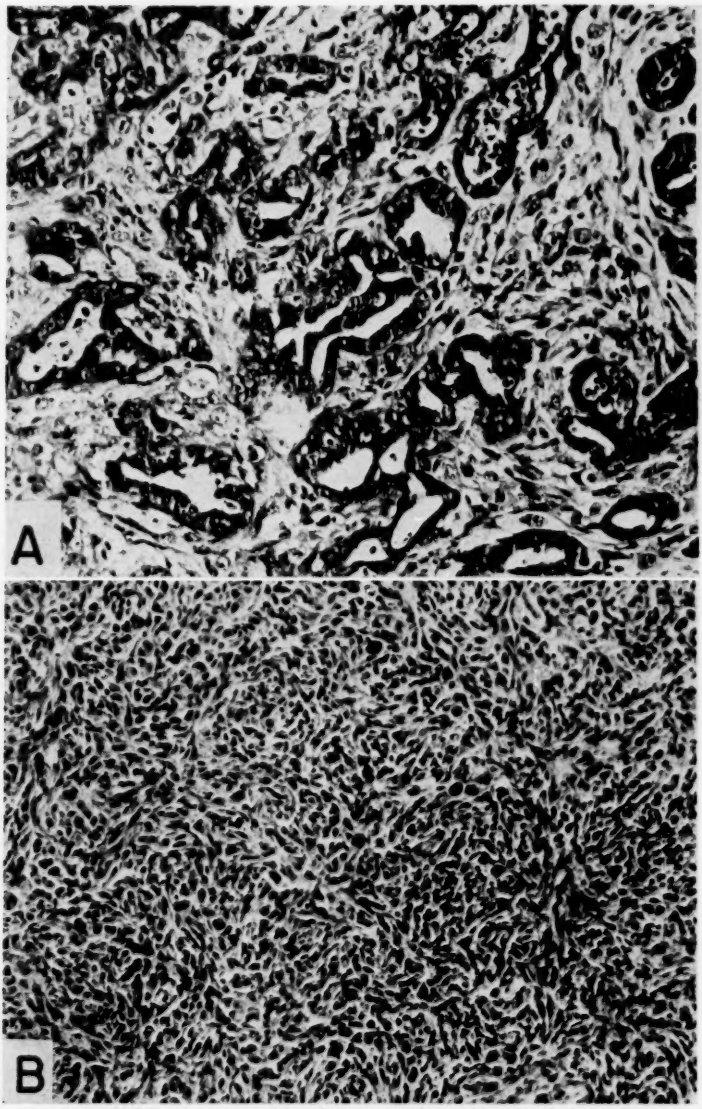

FIG. 2.—(*A*) Mixed tumor arising from transplantation of cells from clone 4-2, showing area of epithelial differentiation into tubular structures. H &E, ×143. (*B*) Well-differentiated fibrosarcoma arising from transplantation of cells from clone 4-2. Several multinucleated giant cells are present. H & E, ×143.

of at least one virus-specific substance, namely, the specific complement-fixing antigen present in tumors and transformed cells of various species origin.[9] This antigen may be identical to the transplantation antigen demonstrated in SV40 hamster tumors.[10–12] Whether the genome is associated with host-cell chromosomes, as in bacterial lysogeny, is not known.

In similar studies of clones of human cells transformed by SV40, Pontén *et al.*[13] found virus in a high proportion of clones. Their results are difficult to compare with ours, however, since the cloning was not done in the presence of antiserum, and the overlay procedure was not used.[5, 6]

Transplantation of the clonal lines indicated that a single epithelioid transformed cell is capable of differentiating into cells with either fibroblastic morphology or epithelial cells capable of forming tubular structures. The fibroblastic clone 3-3 gave rise only to sarcomatous tumors.

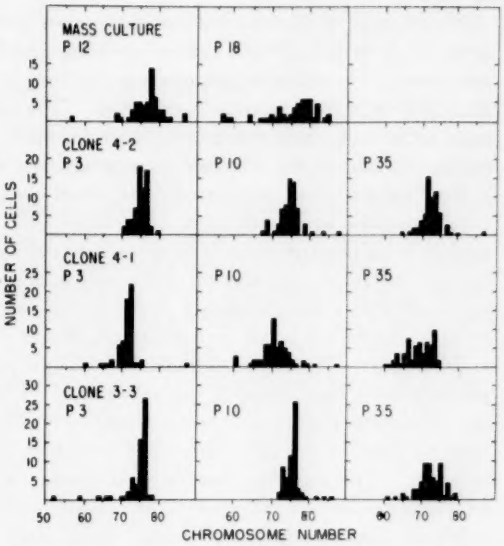

FIG. 3.—Chromosome-number distributions of cells from THK-1 line and from three clones derived by single-cell isolation. Passage numbers are indicated.

The induction of mixed tumors by clonal lines lends support to the concept that the initially transformed cell may be of a single-cell type. This may be primordial mesenchymal cell which serves as the precursor of both the fibroblastic and epithelial cells. It is of interest that the avian renal nephroblastoma, a mixed tumor composed of epithelial and fibrosarcomatous elements resulting from infection with the avian leukosis virus, develops from primitive nephrogenic mesenchyme residual in the postembryonic kidney.[14, 15] The hamster kidney is incompletely developed at birth, and nests of these mesenchymal cells are interspersed between the tubules.[16] Although transformation might occur in these elements in embryonic kidney monolayers, it is doubtful that such cells would be present in the kidneys of 7-week-old hamsters.

Since both tubular epithelium and stromal tissue develop from the primitive, mesenchymal cells that comprise the metanephric anlarge,[17] an alternative hypothesis would be that infection of either tubular or stromal elements might result in a disturbance of differentiating mechanisms with subsequent morphologic variability.

The cytogenetic findings are of interest when compared to the histopathology of these tumors. While all three clones ultimately underwent deterioration of the original stem line, the rate at which this occurred differed. Clone 3-3 appeared to maintain its modal chromosome number relatively well for at least 10 passages. Clone 4-1 showed rapid deterioration beginning with 3 passages, while clone 4-2 was intermediate. Thus, at the time of animal inoculation (5th passage), clone 3-3 still had a strongly predominating modal chromosome number while clones 4-1 and 4-2 had begun to deteriorate karyotypically. It is conceivable then that the number of cell types produced on transplantation was related to the degree of instability of the karyotype.

The efficacy of the cloning technique used here for the isolation of clones derived from single cells is always subject to some question. Although no clumps of cells were seen in the examination made at the time of plating, the possibility that a few clumps were present cannot be excluded. The relative homogeneity of chromosome number in each clone when first examined speaks for their single-cell origin. The interpretations of the virologic data presented here would not be affected seriously if the "clones" had originated from small clumps. Concerning the induction of mixed tumors by clonal lines, the clones (4-1 and 4-2) which gave rise to such tumors were homogeneous with respect to cell type, and thus would not appear to be derived from mixtures of epithelioid and fibroblastic parent cells. Clone 3-3, which showed most variability in cell morphology in the early passages and would be most suspect in being derived from a clump of cells, gave rise to pure sarcomata.

Summary.—Three clonal lines, two of epithelioid and one of fibroblastic morphology, were established in the presence of SV40 antiserum from a continuous cell line of hamster kidney cells transformed by SV40. Virus was consistently recovered from all three clones only when they were planted directly on Cercopithecus kidney cultures. This suggests that the SV40 genome is integrated in the genetic apparatus of a high proportion of transformed hamster kidney cells.

Mixed tumors (carcinosarcomas) arose after transplantation of the epithelioid clones, indicating that a transformed cell may differentiate into cells with either an epithelial or fibroblastic morphology.

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*THE EFFECT OF MITOTIC CYCLE DURATION ON CHROMOSOME BREAKAGE IN MERISTEMATIC CELLS OF PISUM SATIVUM**

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One of the more apparent differences between acute and chronic irradiation is that exposure in the former is generally confined to a small fraction of a single mitotic cycle while, in the latter, exposure of mitotically active tissue usually involves at least several cycles. Because of this difference, the number of cells in each stage of interphase would be of primary importance in acute radiation experiments since radiosensitivity is not the same in the G_1, S, and G_2 periods.[1,2] G_1 is that portion of interphase preceding DNA synthesis, S is that portion of interphase in which DNA synthesis occurs, and G_2 is that portion of interphase following DNA synthesis and preceding mitosis. The duration of the total mitotic cycle should be more important in chronic experiments because most of the cells in the tissue will have passed through each of the interphase stages (G_1, S, and G_2) during the period of irradiation, thus negating any differential effect.

Earlier experiments designed to evaluate the influence of mitotic cycle time in chronically irradiated tissue showed that, at equivalent daily exposure rates, more chromosome damage per cell was produced in meristems grown at low temperatures which presumably had longer cycle times.[1,2] From these observations it was hypothesized that with progressively longer mitotic cycle times, progressively greater amounts of radiation damage accumulated per nucleus in chronically exposed proliferating cells. The experiments described in this paper were designed to investigate further the influence of cycle time in chronically irradiated tissue.

Material and Methods.—Pea seedlings (*Pisum sativum*, var. Alaska) were germinated at approximately 21°C for three days in vermiculite moistened with distilled water. When the seedlings had developed primary roots of 2–3 cm in length they were placed in plastic containers in Hoagland's nutrient solution. The plastic walls were about 1.5 mm thick, and depth of the nutrient solution in the container was about 0.6 cm; thus, shielding due to the nutrient solution varied from something less than 0.6 cm to almost none. Therefore, even the maximum amount of shielding would have a very small effect on the dose of gamma radiation absorbed by the tissue. In each experiment the seedlings were exposed to a Co^{60} source for 20 hr per day for three days at several different exposure rates. An exposure period of three days was selected because previous experiments had shown that both the number of cells in the root meristem and the root growth rate showed a measurable radiation effect at this time with most of the dose rates used.[3]

Seedlings in containers with aerated nutrient solution were placed in a controlled-environment chamber throughout the period of irradiation. Experiments were performed in the dark at 10°, 15°, 25°, and 30°C, the variation in temperature being about ±1.0°C. The doors of the chamber were opened randomly and intermittently to expose the seedlings to light, thus eliminating the possibility of photoinduced synchrony of cell division. Control seedlings were grown under similar conditions in a growth chamber which received no gamma radiation.

The technique used for measuring the duration of mitotic cycles involved the production of a tetraploid (4x) population of cells in diploid roots.[4,5] The tetraploid or tagged cells were produced at 30° and 25°C by a 30-min treatment and at 15° and 10°C by a 60-min treatment with colchicine at a concentration of 5×10^{-4} *M*. The affected cells carry out karyokinesis but no cytokinesis, and then proceed into interphase in the 4x condition. The time between the production of these 4x cells and their appearance in the following division is a measurement of the minimum mitotic

cycle time. Any changes produced by various treatments can be detected by comparing the characteristics of the control 4x population with that of the treated cells.

The data from these experiments are expressed in the form of a polyploid index, which is the number of dividing tetraploid cells (prophase through late anaphase) per thousand cells. The minimum mitotic cycle time was defined to be the time, to the nearest hour, that the polyploid index reached a value of 10. Each index is the average of data from three slides, each slide containing one meristem. The meristem in this case refers to the vividly Feulgen-stained portion of the root.[3] One thousand cells were scored per slide.

To obtain an estimate of the amount of damage produced from exposure to radiation, chromosome aberrations were scored at anaphase on Feulgen-stained squashes of meristems (the terminal 2 mm). Fifty anaphases were scored from each of three meristems per exposure rate.

Results. –The influence of temperature on mitotic cycle time, as measured by the colchicine technique, substantiated earlier observations made on *Pisum*.[6] In general, the mitotic cycle time showed considerable temperature dependence between 10°–25°C. This temperature dependence was no longer apparent at 30°C. The effect of three days of gamma irradiation on the minimum cycle time was generally statistically insignificant (Table 1 and Fig. 1). However, in two instances the

TABLE 1

SUMMARY OF EXPERIMENTAL RESULTS

Temperature (°C)	Approx. min. mitotic cycle time (hr)*	r/day	Accumulated exposure (r) in 3 days (A)	Max. number of cycles in 3 days (B)	r/cycle A/B	Per cent abnormal anaphases†
30	10	0	0	7	0	0
	10	250	750	7	107	15.0 ± 2.35
	10	500	1,500	7	214	25.7 ± 1.6
	10	1,000	3,000	7	429	57.5 ± 2.96
25	10	0	0	7	0	0
	10‡	185	555	7	79	10.0 ± 0.82
	10	250	750	7	107	15.5 ± 2.67
	10	500	1,500	7	214	23.5 ± 1.51
	10	1,000	3,000	7	429	54.3 ± 3.28
15	22	0	0	3.3	0	0
	22‡	185	555	3.3	170	20.0 ± 1.56
	22	200	750	3.3	230	28.8 ± 4.5
	22	500	1,500	3.3	460	48.0 ± 1.43
	—	1,000	3,000	—	920§	77.33 ± 2.5
10	34	0	0	2.1	0	0
	34‡	40	120	2.1	5.7	6.0 ± 1.65
	34	85	255	2.1	120	9.5 ± 1.41
	—	325	975	—	459§	52.6 ± 0.66

* The time to the closest hour at which the control polyploid index (P. I.) reached a value of approximately 10.
† Rate of abnormal anaphase production = 0.119/r/cycle (see text).
‡ Estimated minimum mitotic cycle times at these exposure rates.
§ Value assuming no increase in cycle time.

cycle time appeared to be longer than the control. When the cycle time was increased to approximately 22 hr by lowering the temperature to 15°C, the cells exposed to 1,000 r/day showed a longer minimum cycle time as compared with the control. Likewise, when the minimum cycle time was about 34 hr (at 10°C), those cells exposed to 325 r/day also showed an increased minimum cycle time when compared with the control.

The influence of mitotic cycle duration on the production of cells having chromosome aberrations at anaphase is shown in Table 1 and Figure 2. It will be noted that when the data are expressed as the percentage of abnormal anaphases versus exposure per day, a large difference was observed between the percentage of abnormal cells produced per unit of exposure in experiments in which the proliferating

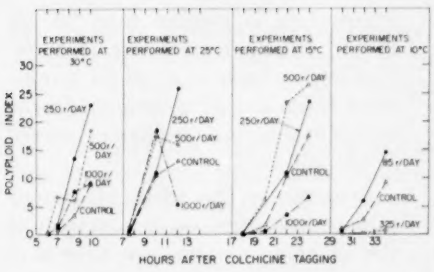

FIG. 1.—Minimum mitotic cycle measurements in meristems at different temperatures after three days of chronic gamma irradiation. Minimum cycle duration was defined as that time, to the nearest hour, that the polyploid index reached a value of 10.

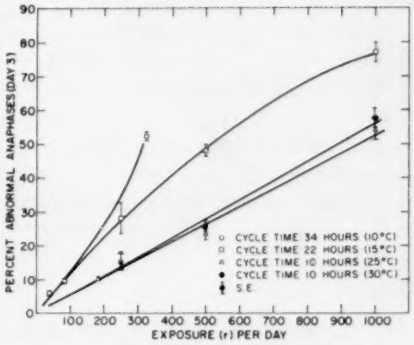
PERCENT ABNORMAL ANAPHASES (DAY 3)
EXPOSURE (r) PER DAY
CYCLE TIME 34 HOURS (10°C)
CYCLE TIME 22 HOURS (15°C)
CYCLE TIME 10 HOURS (25°C)
CYCLE TIME 10 HOURS (30°C)
S.E.

FIG. 2.—Percentage of abnormal anaphases after three days of chronic gamma irradiation at different temperatures and various exposure rates.

cells had large differences in cycle time. For instance, in the curves of Figure 2 at 300 r/day for three days, cells having a minimum cycle time of about 34 hr had an intercept value of approximately 45 per cent abnormal anaphases, while those having minimum cycle times of 22 hr and 10 hr had 31 and 16 per cent abnormal anaphases, respectively.

The data illustrated in Figure 2 suggested that in the case of chronic irradiation either an increased mitotic cycle duration or the lowering of the temperature would produce more cells with chromosome aberrations. The influence of temperature, although not previously mentioned, should be considered as a possible explanation for these observations, for it is generally known from studies with acute irradiation that the frequency of chromosome aberrations under aerobic conditions usually increases with a decrease in temperature.[7–9] Therefore, it seemed appropriate to attempt to separate the influence of the mitotic cycle duration and the effect of temperature, assuming that they both operate under the conditions described. These two variables could be evaluated if the percentage of abnormal anaphases were expressed as a function of gamma-ray exposure in terms of r per cycle. If the number of abnormal cells was dependent on cycle time only, a relatively smooth curve should be produced. If, on the other hand, temperature were the more influential factor, the experimental points from the observations made at 10°C should be above those of 15°C, etc. The net result would be a family of curves differing only in slope. The value of the slope would increase with decreasing temperature. Figure 3 shows the resultant curve when the percentage of abnormal anaphases after three days of exposure is expressed as a function of exposure per mitotic cycle. The curve is linear until about 50 per cent of abnormal anaphases is observed. A family of curves is not indicated in Figure 3; therefore the differences observed in Figure 2 are considered to be primarily the result of different cycle times and not a decrease in temperature. The effect of an increase in oxygen concentration due to the lowering of the temperature may also be precluded. Reading from the curves in Figure 2, the abnormal anaphases at 300 r/day are 45 and 16 per cent, respectively, for 10° and 30°C. The percentage increased by a factor of 2.8. For water, the oxygen concentration increases from 33.6 to 34.4 per cent (a factor of 1.02) as the

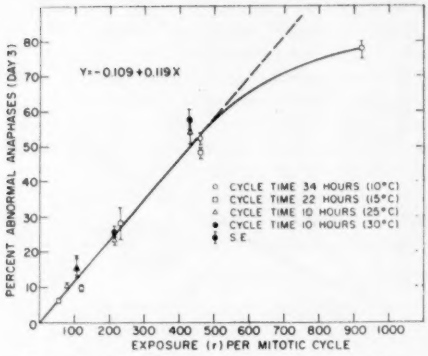
PERCENT ABNORMAL ANAPHASES (DAY 3)
Y=-0.109+0.119X
CYCLE TIME 34 HOURS (10°C)
CYCLE TIME 22 HOURS (15°C)
CYCLE TIME 10 HOURS (25°C)
CYCLE TIME 10 HOURS (30°C)
S.E.
EXPOSURE (r) PER MITOTIC CYCLE

FIG. 3.—Percentage of abnormal anaphases after three days of chronic gamma irradiation versus roentgens per cycle.

temperature is lowered from 30° to 10°C. Minimum cycle time, however, changes by a factor of about 3.4, a value much closer to that determined for the change in abnormal anaphases observed at 10° and 30°C.

Discussion.—The effect of chronic gamma irradiation on the duration of the minimum mitotic cycle time is of considerable interest. No statistically significant difference in minimum mitotic cycle time was observed following three days of chronic gamma irradiation at 25° and 30°C. These observations substantiated earlier experimental results performed at 20°C.[3] However, as the cycle duration was increased from about 10 hr at 30° and 25° to approximately 22 and 34 hr at 15° and 10°, respectively, it became apparent that an increase in cycle time due to chronic gamma irradiation depended on the total exposure per cycle. As long as this value remained below a critical level, no cycle time alteration was observed. For *Pisum sativum* this value was estimated to be in the vicinity of 440–450 r/cycle, since 459 r/cycle at 10°C produced an increase in mitotic cycle time, and 429 r/cycle at 25° and 30°C showed no effect.

Generally, it has been observed that with acute irradiation the aberration frequency increases with decreasing temperature under aerobic conditions (cf. ref. 10 for a recent review of this and other aspects of chromosome aberrations). The absence of a temperature effect when cycle time is considered raises the following question concerning the results of acute experiments: does the mitotic cycle time play a role in producing the increase in aberrations with decreased temperature? The experimental results described in this paper show that lower temperatures increase the mitotic cycle time. Also, as shown by Wimber,[11] the interphasic periods of G_1, S, and G_2 are altered disproportionally by a decrease in temperature. Thus, it is possible that the general observation of higher aberration frequencies with a decrease in temperature with acute exposures could be the result of elongated G_2 and/or G_1 periods at the expense of S. Such an alteration would increase the chromosome breakage frequency because breakage in G_2 and G_1 periods is greater than in the S period.[12] Therefore, the expected consequence would be an increase in the aberration frequency with decreasing temperatures as observed.[7–9] Perhaps the absence of temperature effect in chronically irradiated *Pisum* cells can be attributed to the fact that expression of the data in terms of roentgens per cycle somehow corrects for any disproportionate changes in the interphasic periods as well as for the effect of any additional time for reconstitution of chromosome breaks.

Determination of the frequency of abnormal anaphases was made after three days of chronic gamma irradiation. Therefore, it can be assumed from observations made with *Vicia faba*[13] that a steady-state condition had been established with respect to the rates at which damaged cells were produced in and removed from a

population of proliferating cells exposed to a given dose rate. A model of this steady-state condition is illustrated in Figure 4. K_1 is the rate at which damaged cells are produced, and K_2 is the rate at which damaged cells are removed from a proliferating population, P.

Presumably, the frequency of abnormal anaphases observed after three days of irradiation was the result of the relative values of K_1 and K_2. This would mean that the observed rate (K_3) at which abnormal anaphases were produced is equal to the algebraic sum of K_1 and K_2, or

$$K_3 = (K_1) + (-K_2).$$

It is obvious that K_2 must be less than K_1 if abnormal anaphases are to exist. This leads to the question concerning the relative values of K_1 and K_2. Inspection of the curve in Figure 3 should provide a clue to the answer. The first portion of the curve is linear; therefore, K_1 and K_2 are increasing proportionally. At about 420–450 r/cycle, K_2 is rapidly approaching K_1, so that no net increase in abnormal ana-

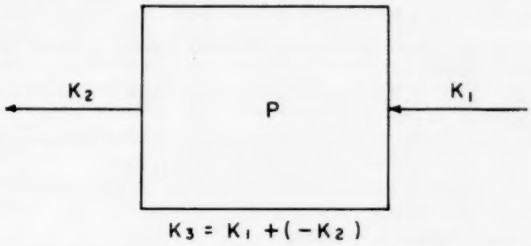

FIG. 4.—A model illustrating a steady-state condition in chronically irradiated root meristems. P is a population of proliferating cells, K_1 the rate at which cells with damaged chromosomes are produced, and K_2 the rate at which the damaged cells are removed from P. K_3 is the algebraic sum of K_1 and K_2, and is the observed rate at which abnormal anaphases are produced.

phases would be expected at very high exposure values. A possible explanation for a rapid increase in K_2 is that, at exposure rates exceeding 450 r/cycle, cells with chromosome breaks had accrued sufficient radiation damage to prevent mitosis and hence the breaks would not be scored.

Evaluation of K_3 was obtained from the regression line for the linear portion of the curve in Figure 3. The equation is

$$\% \text{ Abn. anaphases} = 0.119 \text{ (r/cycle)} - 0.109.$$

The slope of the regression line estimates K_3. For *Pisum* the slope is 0.119/r/cycle; thus,

$$K_3 \cong 0.119/\text{r/cycle}.$$

It is apparent that the hypothesis stating that an increased mitotic cycle time increases the production of damaged cells is not valid when exposure/cycle is considered rather than exposure/unit time (e.g., hours or days). Figure 3 clearly indicates that an exposure of 200 r/cycle to a group of cells dividing every 10 hr will produce approximately the same result as 200 r/cycle to a group dividing every

20 hr. The same would not be true, however, if both groups of cells were exposed to 200 r/day.

Summary.—Experiments were performed with seedlings of *Pisum sativum* to test the hypothesis that radiosensitivity with respect to chromosome damage increases during chronic irradiation with increased mitotic cycle time. Minimum cycle times were determined for root meristem cells. Temperatures of 30°, 25°, 15°, and 10°C were used to obtain different minimum cycle times. The results of chronic exposures indicated that (1) the minimum cycle time was not altered in *Pisum sativum* until the total exposure per cycle exceeded about 440–450 r; (2) the percentage of cells showing damaged chromosomes at a stated daily exposure rate increased with decreasing temperatures if mitotic cycle time was not considered; but (3) when the cycle time is taken into account, the production of damaged cells is a linear function of the amount of total exposure per mitotic cycle up to the critical value 440–450 r/cycle.

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*TREE LINES AND GERM PLASM—A STUDY IN EVOLUTIONARY LIMITATIONS**

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A rough estimate of the capacity of plants to adjust to extreme environments can be obtained through a survey of the location of tree lines in different parts of the world. This is because trees are subject to severe stresses from climate.

Definition of Tree Line.—Tree lines and alpine vegetations are contiguous. Where the trees disappear, the alpines begin, rich in growth forms of the low-bush

and cushion types, known as elfinwood or Krummholz. The tree line is where a tree species disappears or changes into its elfinwood race.

Northern Hemisphere botanists usually have in mind a tree line of northern conifers. It has generally been accepted as a fact that the tree line rises toward the low latitudes. This assumption is valid only until about mid-latitude, where our northern conifers disappear. The germ plasm of the tree species is actually a highly important factor in determining its limits of growth.

Factors That Influence Growth of Trees.—Data from all over the earth indicate that low shrubs and herbs have advanced farther toward high latitudes and high altitudes than have trees of the same genus or species. This fact suggests that unfavorable climates impose more severe limitations upon trees than upon shrubs and herbs.

Trees face physical and physiological stresses in their upward transport of water from the ground, and the stresses increase with the height of the trees. The environment influences the water balance of trees: uptake of water is slowed down with lower temperatures, and loss of water through transpiration is increased at higher temperatures, in stronger wind, and under dry air conditions. Low temperatures can produce drying effects similar to those caused by drought; trees native to tropical latitudes, such as the coffee tree, may therefore show frost symptoms before the freezing point has been reached. Dry, dead tops of trees are common at windy ocean shores, in arid regions, and in cold, wet ground at high latitudes and altitudes. Visible frost symptoms may therefore result from many causes.

Heredity influences the degree of tolerance to frost. Hagem[9] tested seedlings of *Picea sitchensis* (Bong.) Carr from coastal areas of western North America in outdoor sowings near Bergen, western Norway, at 60° North latitude. During successive years 94% of seedlings from latitudes 47° North in the state of Washington were damaged by frost, in contrast with only 7% from latitudes 55–58° in southern Alaska. The introductions from intermediate British Columbia had intermediate frost susceptibility, only 64% of their seedlings being damaged at Bergen. From this experiment it is obvious that Sitka spruce is composed of climatic races that differ in their susceptibility to frost.

Inherited Responses That Mitigate Water Stresses in Trees.—Trees buffer the stresses that extreme environments impose upon their water balances by developing adjustments such as: (1) low growth forms (elfinwood), effectively reducing evaporation through snow covering; (2) reduced leaf surfaces, as in most conifers; (3) dropping of the leaves during the unfavorable season, accomplished through complicated growth processes; and (4) physiological compensations (very little is known about such adjustments in trees). These four groups of adjustments relate to the germ plasm and are regulated by genes.

Combinations of such responses mitigate the stresses but do not enable a tree to grow everywhere on the earth. By far the greater percentage of tree species have therefore remained within the favorable tropical regions, and only very few plant families have evolved trees adjusted to the extremely cold climates at high latitudes and high altitudes.

Examples of Contrasts in Germ Plasms.—During 1953 the author was given an opportunity to compare low-latitude montane and alpine Brazilian vegetations with similar ones at higher latitudes and altitudes in the Sierra Nevada of California.[2] (This visit was made possible by a cordial invitation from the Cultural Division of the Brazilian Ministry of Foreign Relations as a part of the Brazilian program for international exchange of scholars.) These two regions are botanically as different as any two on earth; in both, the montane ranges are forested, and alpine vegetations have evolved from local sources.

At the 22° South latitude in Serra do Mar of the state of Rio de Janeiro, the transition from the forest to the alpine planalto occurs at approximately 1,950 m altitude. This change was observed at two places: in the 2,300-m-high Serra dos Orgãos (Organ Mountains) and 100 km farther eastward at Mt. Itatiaía (highest in Brazil,

TABLE 1

VEGETATION OF SERRA DOS ORGÃOS NATIONAL PARK, SOUTHEAST BRAZIL, AT 22° LATITUDE*

Family groups (orders), by Englerian numbers	Number of Species: Trees	Total, woody plants	Total, all kinds
Coniferae	—	—	—
Monocotyledonae:			
4. Glumiflorae: panicums, cortaderias (no Carex)	—	4	35
5. Principes: palms	6	6	6
8. Farinosae: bromeliads, Xyridaceae, eriocaulons	—	—	59
9. Liliiflorae: amaryllis, vellozias, dioscoreas (no lilies; no rushes)	—	3	15
11. Microspermae: orchids, burmannias	—	—	226
Dicotyledonae—Chloripetalae:			
2. Piperales: pepper relatives	1	27	28
3–11. Willows, walnuts, birches, oaks, beeches, chestnuts	—	—	—
12. Urticales: figs, mulberry, nettle, elm relatives	12	13	18
13. Proteales: roupalas	7	7	7
18. Ranales: laurels, drimys, buttercups, etc.	11	49	51
21. Rosales: saxifrages, cunonias, roses, legumes	40	57	73
23. Geraniales: cocas, simarubes, mahoganies, malpighias, euphorbias	21	53	84
24. Sapindales: hollies, cupanias (no maples)	16	26	28
25–26. Rhamnales, Malvales: gouanias, sloaneas, mallows	5	10	22
27. Parietales: camellias, guttifers, violets, flacourtias, begonias	22	38	80
28. Opuntiales: cacti (predominantly epiphytes)	—	—	14
29. Myrtales: myrtles, melastomes	82	204	214
Dicotyledonae—Sympetalae:			
1. Ericales: clethras, heathers, blueberries	4	24	24
6. Tubiflorae: borrages, verbenas, labiates, nightshades, figworts, bignonias	23	74	171
8. Rubiales: camphor and coffee relatives	29	58	84
10. Campanulatae: lobelias to sunflowers	10	61	142
21 orders, subtotal	289	714	1,381
14 other orders	29	30	194
35 orders, 129 families, total	308	744	1,575
	19.5%	47.2%	

* Altitudes, 925–2,300 m. Tree line at 1,950 m.

2,800 m). To a Northern Hemisphere botanist this is an incredibly low tree line at that latitude.

Serra dos Orgãos was visited in company with Dr. C. T. Rizzini of the Rio de Janeiro Botanical Garden, to whom I owe gratitude for many courtesies. After intensive field studies he published a complete list of the plants within the park in 1954.[29] Altitudes vary between 925 and 2,300 m, providing a rugged terrain in the area composed of gneiss and granite estimated to be approximately 160 km². The lower slopes are covered by dense cloud forests that are replaced by an alpine bush and grass vegetation at *ca.* 1,900–2,000 m.

An analysis of the Organ Mountain vegetation has now been made (Table 1). It has 286 species of ferns and allies and 1,575 species of flowering plants that belong to 129 families. Most of the orders to which the most important northern temperate forest trees belong are absent.

In common with other forests at low latitude, the Organ Mountain forests have a high percentage of tree species and woody climbing shrubs. There are 308 species of trees in addition to 21 species of tree fern, but none of them go above the 1,950-m level. As indicated by Table 1, these trees are members of tropical orders and families. The myrtle relatives alone constitute 26 per cent of the trees.

The alpine vegetation above the 1,950-m level is composed of grasses and low bushes, such as a small bamboo, a holly, an acacia, a baccharis, and myrtle and heather relatives. These resemble alpines of the higher-latitude families but are related to the trees in the forest lower on the mountain.

The last trees before the alpine vegetation are such as *Drimys brasiliensis* Miers of the Winteraceae, *Belangera* and *Weinmannia* of the Cunoniaceae, and *Ternstroemia* of the Camellia family. They show the crippling effects of occasional light frosts but nevertheless are among the hardiest of the local tree species. They belong to families that have not evolved trees within the temperate zones.

Climatic records are available from the 1,025-m level within the cloud forest (Table 2, left),[29] indicating that the lowest temperatures (7–10° C) during the winter months are well above the freezing point. The center columns indicate the temperatures at 2,200 m on Mt. Itatia´a,[1] slightly above the cloud forests and within the alpine planalto vegetation; during the winter the monthly mean minimal temperatures are 5–11° C, well above freezing, but slight night frosts and snows occur occasionally, as indicated by lowest temperatures. All the 308 tree species are absent above this point, although the temperatures compare with those in the California outer Coast Ranges, where such trees as *Sequoia sempervirens* are native and grow to giant size.

In sharp contrast with the Brazilian forests is the situation at the 3,000-m altitude in the Sierra Nevada of California. Three large tree species thrive here in a climate (Table 2, right) where the monthly average minimal temperatures remain below the freezing point during eight months of the year, and frost can occur any day during all seasons. The trees survive temperatures as low as −30° C, and must possess germ plasms of a remarkable hardiness.

These three California trees are the lodgepole pine, *Pinus murrayana* Balf., a relative of the North European Scotch pine; the whitebark pine, *P. albicaulis* Engelm., a relative of *Pinus cembra* L., the tree-line tree of the Swiss Alps and the Altai Mountains; and the mountain hemlock, *Tsuga mertensiana* (Bong.) Sarg., which has close relatives across northern North America and at high altitudes in Japan and in the Himalayas.

TABLE 2

TEMPERATURES (IN °C) IN REGIONS OF TWO CONTRASTING VEGETATIONS

	South: 22° latitude					North: 38° latitude				
	Southeast Brazil, Serra do Mar					California, Sierra Nevada				
Altitude:	1,000 m		2,200 m			3,000 m				
Precipitation:	318.3 cm		241.7 cm			78.3 cm				
	Cloud forest		Alpine scrub (planalto)			Subalpine forest				
	308 tree species		No tree species			3 tree species				
Months	Lowest	Highest	Lowest	Mean minima	Mean maxima	Months	Lowest	Mean minima	Mean maxima
July	7.1	21.6	−6.0	5.1	13.0	Jan.	−31.7	−11.8	1.7
Aug.	8.7	23.4	−3.4	6.0	14.3	Feb.	−27.8	−11.8	2.8
Sept.	10.4	23.7	−5.0	8.0	15.7	March	−26.1	−9.6	4.8
Oct.	11.5	25.7	−1.1	8.0	15.2	April	−23.4	−6.9	7.0
Nov.	12.5	23.7	−0.3	8.8	15.9	May	−20.0	−3.3	10.6
Dec.	12.3	24.3	2.4	10.2	16.2	June	−11.7	1.2	15.3
Jan.	18.8	28.2	3.7	11.0	16.7	July	−3.3	5.7	20.0
Feb.	14.8	29.0	2.4	11.0	16.8	Aug.	−1.7	5.4	19.7
March	14.6	27.3	2.2	10.8	16.4	Sept.	−11.1	1.6	15.6
April	11.8	23.1	0.3	8.7	15.2	Oct.	−19.4	−2.2	10.6
May	9.6	23.0	−3.0	6.6	13.9	Nov.	−22.2	−6.2	6.2
June	9.5	21.7	−4.8	5.8	13.4	Dec.	−27.2	−9.3	3.1

The three Sierra Nevada conifers have their tree limits at slightly different altitudes and exposures of 3,075–3,330 m. They develop giant trees almost to their tree lines, although within 50–75 m below the tree limits, each species is composed of multitudes of interspersed biotypes that range from low, horizontal elfinwood cushions through many intermediates to fully erect, large trees. Only the low elfinwoods of each remain beyond the tree line. The whitebark pine has the highest tree line of these three, at 3,300 m. Some of its elfinwood plants have a spread of up to 20 m with a height of only 1 m.

The Group of Most Tolerant Tree Species.—The striking difference in tolerance between the Brazilian low-latitude forest and the extremely tolerant tree species of the high Sierra Nevada prompted a search through regional floras and other botanical literature[6, 8, 15–17, 22, 24–26, 32, 33] to discover those species that provide the tree outposts toward the high latitudes and altitudes. This search produced some rather unexpected facts, which are listed below:

(1) Only about 12 species complexes have been able to evolve trees for extremely cold climates; (2) a tree species occupying extreme habitats in one part of the earth is likely to have one or several close relatives under different names in similar extreme habitats of the same altitudinal zone; (3) if a tree species grows at high latitudes, it can also be expected to grow at high altitudes farther south in the temperate zone; and (4) climatically highly tolerant species belong to taxonomically "critical" species complexes. Many transitional forms, interpreted as being natural hybrids, tend to obscure the limits between the species. Controlled hybridization experiments[7, 10–13, 18, 19, 21, 27, 28, 31, 35] have also shown that the species of most of the 12 complexes intercross fairly freely, so that each species complex represents a large gene pool.

The twelve highly tolerant species complexes occupy a broad belt between the latitudes of approximately 35–68° North or occasionally to the 70th latitude; toward their south limits these species ascend to 3,000–3,700 m altitudes of high

TABLE 3

MOST TOLERANT TREES, 12 SPECIES COMPLEXES (NORTHERN HEMISPHERE, HIGH LATITUDES, 35–70° NORTH)

	Europe	Asia	North America
Pinales, all n = 12:			
1. Larches:		*Larix decidua*—*sibirica*—*gmelini*	*laricina* →
2. Pines:		*Pinus silvestris* →	*contorta*—*murrayana*—*banksiana*
		montana—*mughu*	
3.		*Pinus cembra* → *pumila*	*albicaulis*
4. Spruces:		*Picea excelsa*—*obovata*—*alcocquiana*	*sitchensis*—*glauca*—*mariana*
		smithii	
5. Firs:		*Abies nordmanniana* → *veitchii*	*lasiocarpa* → *balsamea*
6. Hemlocks:		*Tsuga* → *araragi*	*mertensiana* → *canadensis*
		brunoniana	*heterophylla*
Cupressales, n = 11:			
7. Junipers:		*Juniperus communis* →	*communis* →
Salicales, n = 19:			
8. Poplare:		*Populus tremula* →	*tremuloides* →
9. Willows:		*Salix phylicifolia*—*pyrifolia*—*pulchra*	*pulchra*—*arbuscula*
Fagales, n = multiples of 7 in these:			
10. Birches:		*Betula tortuosa*—*cajanderi*—*ermani*	*kenaica*—*papyrifera* →
11. Alders:		*Alnus incana* → *fruticosa*	*crispa*—*incana* →
Rosales, n = 17 in this complex:			
12. Mountain ash:		*Sorbus aucuparia* → *sambucifolia*	*scopulina*—*americana decora* *sitchensis*

mountains. Table 3 lists the members of the 12 complexes with the European members to the left, the Asian in the center, and the North American ones to the right. The species names within each complex serve as guides to the literature. The gametic numbers of chromosomes[5] are indicated.

Six of the members of this highly tolerant group are pine relatives, all having a uniform number of 12 pairs of chromosomes. Another conifer is the circumpolar *Juniperus communis* L., $n = 11$, which has both erect trees and elfinwood forms. Two complexes belong to the willow family: the aspens, *Populus tremula-tremuloides*, $n = 19$, and relatives of *Salix phylicifolia* L., having multiples of $n = 19$. Two others belong to the birch family, a polyploid complex of birches, *Betula*, and the alders, *Alnus*. Finally, there are the mountain ashes, members of the *Sorbus aucuparia* complex of the rose family, $n = 17$.

These are all Northern Hemisphere plants. In the Southern Hemisphere no trees exist at comparable combinations of altitudes and latitudes. At least 7 of the 12 complexes have at one point or another evolved elfinwood forms beyond their tree line.

Trees of Medium Tolerance.—A much larger group of a few thousand tree species have moderate tolerances and are natives between the latitudes of 20–50°, primarily in the Northern Hemisphere (Table 4). This group is characterized by the hundreds of species of oaks, beeches, and chestnuts of the oak family,[23] having uniformly 12 pairs of chromosomes and prevalent hybridity. With the oaks are associated the yellow pines and the cedars, equally hybridizing. Compared with the Brazilian low-latitude group of trees, the oaks, yellow pines, and cedars have respectable cold tolerance, but they are definitely more tender than the trees of the northernmost group.

The highly tolerant and medium tolerant groups of trees overlap many latitudes. In central California, for example, *Quercus kelloggii* Newb. marks the oak tree line at 2,000 m in the Sierra Nevada; it is followed by an elfinwood bush species, *Q. vaccinifolia* Kell., which ceases at 2,600 m altitude. The oak tree line is not seen because the conifers of the hardy group go much higher.

One single genus of the beeches, *Nothofagus*, reaches moderate altitudes and fairly high latitudes in the Southern Hemisphere, forming the tree line in southern Chile, New Zealand, and Tasmania. The incense cedars, *Libocedrus*, $n = 11$, are associated with them in the Southern Hemisphere and have one species in North

TABLE 4

MEDIUM-TOLERANT GROUP, EXAMPLES OF TREE SPECIES (LATITUDE 20–55°, BOTH HEMISPHERES)

Coniferae:
- Podocarpaceae: *Podocarpus nubigena*, to 51° S. in Patagonia
- Cupressaceae: *Libocedrus*, Northern and Southern Hemispheres, $n = 11$
- Pinaceae, $n = 12$:
  - Yellow pines: *Pinus ponderosa—jeffreyi—scopulorum*
    hartwegii—arizonica
  - Cedars: *Cedrus atlantica—libani—deodara*

Fagales, $n = 12$ in these:
- Oaks: *Quercus kelloggii*, oak tree line at 2,000 m in Sierra Nevada
  vaccinifolia, elfinwood, to 2,600 m
  Lithocarpus, tree line in Java at 2,700 m (elfinwood)
- Beeches: *Fagus silvatica*, tree line in Italy at 1,830 m (elfinwood)
  Nothofagus, tree lines in Chile, New Zealand, Tasmania

Myrtales:
- *Eucalyptus*, $n = 11$, tree line in Australia at 2,000 m

America. Another conifer, *Podocarpus nubigena* Lindl., has trees until 51° South latitude in Patagonia.[30]

Similar to the oaks of the Northern Hemisphere, one exclusively Australian genus of the myrtle family, *Eucalyptus*, $n = 11$, has evolved multitudes of tree species in many environments. It provides the tree line in the Snowy Mountains at the 35th South latitude at an altitude of 2,000 m, comparing with the tree line of the Kellogg oak in California. Several *Eucalyptus* species have successfully been introduced to the high plateaus around 3,500 m of equatorial regions of other continents where trees are scarce.

Trees of Low Latitudes.—By far the largest number of tree species, probably more than 50,000, occur within the tropics and appear to be "trapped" within the latitudes of about 25° North and 25° South. A sample of this group of trees was discussed under the Serra dos Orgãos vegetation of the southeast Brazilian plateau (Table 1). It would be impossible here to consider in detail the highly varied group of low-latitude trees, and only some of its more tolerant and wide-ranging genera are listed in Table 5.

Some of the more tolerant species of this group transgress to the milder climates of higher latitudes, where they grow near sea level. The three latitudinal groups of trees are obviously somewhat arbitrary and connect by intermediates, but they aid in visualizing great differences in ranges of tolerance.

Multiple Tree Lines.—Because each tree species or tree genus has its own tree line, multiple tree lines can exist. The areas of most tree species are telescoped, however, so that the multiple tree lines are not obvious. Asia Minor has an oak tree line at 925 m, followed by steppe and bush oaks to 1,850 m, in turn replaced by a coniferous forest that produces a coniferous tree line at 2,450 m altitude (ref. 23, p. 405).

In the Argentinian Andes at the latitude of Tucuman,[20, 30] the subtropical evergreen forest has its limits at 1,100 m, followed by a high Patagonian-type deciduous forest of *Nothofagus antarctica* (Forst.) Oerst. that develops a deciduous tree line at 1,850 m. Between 1,850 and 2,750 m is the puna, an "alpine" meadow vegetation of grasses and scattered cushion plants in a region with regular winter snow. Above 2,750 m follows, on the Chilean side, an open *Polylepis* forest with a rosaceous tree line at 3,700 m. Above this "forest" is a second alpine vegetation, the paramo, consisting of low, woody cushions.

TABLE 5

LOW-TOLERANCE GROUP, EXAMPLES OF MOST TOLERANT TREES (LOW LATITUDE: 25° N. to 25° S.)

Coniferae:
- Podocarpaceae: *Podocarpus—Dacrydium* species: Peru,[34] New Guinea, 3,600 m[14]
- Araucariaceae, $n = 13$: *Araucaria* and *Agathis*

Ranales: *Drimys* species: Patagonia;[30] Brazil, 1,950 m;[29] New Guinea, 4,200 m[14]

Rosales:
- Cunoniaceae: *Weinmannia:* Brazil, 1,950 m;[29] Andes,[34] Hawaii, New Zealand
- Rosaceae: *Polylepis leptophylla*: dry Andean tree line, 3,700 m[20, 34]
- Leguminosae: *Sophora:* Andes;[34] Hawaii at 3,050 m; New Zealand

Sapindales:
- Aquifoliaceae: *Ilex:* Brazil,[29] trees in cloud forest; elfinwood above tree line
- Anacardiaceae: *Schinus molle*, high-altitude Andes[34]

Parietales: *Ternstroemia* and *Clusia:* Brazil, tree line at 1,950 m

Rubiales: *Cinchona:* cloud forests, eastern Peruvian Andes, 3,700 m[34]

Evolutionary Limitations within Groups of Species.—Only a few families of plants, such as the relatives of the nettles, roses, legumes, and the violets, have been versatile enough to adjust to almost all latitudes from the tropics to the arctic and antarctic. In doing so, however, we find that the adjustment is accompanied by a change in growth form from trees to herbs.

Most families have remained within certain latitudinal zones. The approximately 5,000 species of myrtle relatives and also many other plant families have remained within the low latitudes, although their genera have crossed oceans and have evolved species adjusted to many local niches within those latitudes.

Similarly, the early progenitors of the pine relatives existed in temperate Europe since early Cretaceous times. Pine relatives still cover the temperate northern latitudes (Table 3), occasionally "spilling over" slightly to lower latitudes, but generally they do not cross the equator. The physiologically adjusted heredities of pine and myrtle relatives have kept them confined within the wide belts of their respective environmental zones during the long geologic periods when they moved around the earth.

The families and orders were classified by taxonomists who used morphological rather than physiological criteria. During the immense evolutionary periods that the tree groups have existed, the cohesive forces of heredity have been strong enough to maintain linkages between morphological and physiological characters. Moreover, mutations that would have radically changed the tolerance ranges were eliminated or prevented.

Strong cohesive forces have been shown to exist within the germ plasms of races, species, genera, and families of plants.[3, 4] Each tree species is highly variable, but its variability does not permit it to be successful in an entirely different zone. A given germ plasm has therefore definite evolutionary limitations beyond which it has not been able to move.

Summary.—The most important factors determining tree growth in various climates are inherited differences in the germ plasm. Each tree species has its own tree line, and some have evolved low elfinwood races beyond the tree limits. The most widely distributed trees belong to species complexes that are known for their ability to hybridize.

The tolerance groups of trees follow major systematic orders. These are based on morphological characters, which apparently are associated with physiological characters of adaptive importance.

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*FUNCTORIAL SEMANTICS OF ALGEBRAIC THEORIES**

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When they introduced the theory of categories in 1945,[1] Eilenberg and Mac Lane suggested the possibility of "functorizing" the study of general algebraic systems. The author has carried out the first steps of this program, making extensive use of the theory of adjoint functors, as introduced by Kan[3] and refined by Freyd.[2] In some directions a very great degree of useful generality can be achieved; for example, the constructions of free algebras, tensor algebras, monoid rings, enveloping algebras of Lie algebras, abelianization of groups, and covariant extension of rings for modules can all be viewed in a unified way as adjoints to "algebraic" functors, and we show that such adjoints *always* exist. Also, by formalizing "semantics" itself as a functor and showing that it has an adjoint, we are able to give a new characterization of equational classes of algebras (viewed as abstract categories) and also to provide a canonical tool for the partial analysis of many nonalgebraic categories and functors.

By an *algebraic theory* we mean a small category $\mathbf{A}$ whose objects are the natural numbers 0, 1, 2, ... and in which each object n is the categorical direct product of the object 1 with itself n times. By an *n-ary operation* of $\mathbf{A}$ is meant any map $n \to 1$ in $\mathbf{A}$. Since n is a product, the projections $\pi_i^{(n)}$: $n \to 1$, $i = 0, 1, \ldots, n-1$ are always n-ary operations for each n in any algebraic theory, but in general there will be more. The maps $n \to m$ in an algebraic theory $\mathbf{A}$ are in one-to-one correspondence with the m-tuples of n-ary operations of $\mathbf{A}$. Any "presentation" of a concept of algebraic structure (e.g., groups, modules over a given ring, Jordan algebras, lattices, etc.), which involves a set of symbols denoting finitary operations together with a set of equations (= identities) relating composite operations, determines an algebraic theory, and conversely every algebraic theory has such presentations. By a mapping between algebraic theories we will understand a functor which preserves products and takes 1 into 1. Algebraic theories and the mappings between them thus form a category $\mathfrak{Z}$.

Each algebraic theory $\mathbf{A}$ determines a large category $\mathcal{S}^{(\mathbf{A})}$ whose class of objects is just the equational class (variety) of all algebras of type $\mathbf{A}$, and whose maps are all (into) homomorphisms between these. An algebra of type $\mathbf{A}$ can be viewed as a product preserving functor $\mathbf{A} \to \mathcal{S}$ from $\mathbf{A}$ to the category of sets; a homomorphism of algebras is then just a natural transformation between such functors. If $\mathbf{A}$ is the algebraic theory whose *only* n-ary operations are projections (i.e., $\mathbf{A}$ is equivalent to the dual of the category of finite sets), then the category of algebras $\mathcal{S}^{(\mathbf{A})}$ is just the category $\mathcal{S}$ of sets. Every map f: $\mathbf{A} \to \mathbf{B}$ of algebraic theories determines in an obvious way a functor $\mathcal{S}^{(f)}$: $\mathcal{S}^{(\mathbf{B})} \to \mathcal{S}^{(\mathbf{A})}$ which preserves underlying sets, i.e., for which $U_{\mathbf{B}} = \mathcal{S}^{(f)} U_{\mathbf{A}}$, where $U_{\mathbf{A}}$: $\mathcal{S}^{(\mathbf{A})} \to \mathcal{S}$, $U_{\mathbf{B}}$: $\mathcal{S}^{(\mathbf{B})} \to \mathcal{S}$ are the underlying set functors (notice the order in which we write composition). We call any functor of the form $\mathcal{S}^{(f)}$ an *algebraic functor*, and we call any category of the form $\mathcal{S}^{(\mathbf{A})}$ an *algebraic category*. Any algebraic theory $\mathbf{A}$ is equivalent to the dual of the full category of finitely generated free algebras in its associated algebraic category.

THEOREM. *Every algebraic functor has an adjoint.*

For example, the category of rings (with unit) and that of monoids are algebraic, and the functor which assigns to each ring the monoid consisting of the same elements under multiplication alone is an algebraic functor. The adjoint to this functor is the well-known construction of the monoid ring. Other instances of this theorem were mentioned in the first paragraph. Note that for f: $\mathbf{A} \to \mathbf{B}$ in $\mathfrak{I}$, the natural transformation from the identity functor on $\mathcal{S}^{(\mathbf{A})}$ to $F\mathcal{S}^{(f)}$, where F is the adjoint of $\mathcal{S},^{(f)}$ need not be a monomorphism (for example, not every Jordan algebra is special—on the other hand every distributive lattice can be embedded in a Boolean ring); a description of those f for which this is so would provide a unified solution to a great number of problems in algebra—for example, when can an algebra be enlarged to contain a root of a given equation?

If we write $\mathbf{A}\mathfrak{S} = U_{\mathbf{A}}, f\mathfrak{S} = \mathcal{S}^{(f)}$, we obtain a functor $\mathfrak{S}$ which we call *algebraic semantics;* the domain of $\mathfrak{S}$ is the dual $\mathfrak{I}^*$ of the category of algebraic theories, and we take as its codomain the category $\mathfrak{K}$ whose objects are functors $U:\mathfrak{X} \to \mathcal{S}$ with arbitrary domain category and with the category of sets as codomain, subject only to the restriction that for each natural number n, the class of all natural transformations $U^n \to U$ is small, where U^n assigns to each X the nth Cartesian power of XU; a map $T:U \to U'$ in $\mathfrak{K}$ is to be any functor $T:\mathfrak{X} \to \mathfrak{X}'$ for which $U = TU'$.

THEOREM. *Algebraic semantics has an adjoint* $\hat{\mathfrak{S}}:\mathfrak{K} \to \mathfrak{I}^*$ *(which we call algebraic structure), and furthermore* $\mathfrak{S}\hat{\mathfrak{S}}$ *is naturally equivalent to the identity functor of* $\mathfrak{I}^*$. *Explicitly, for any* U *in* $\mathfrak{K}$ *the n-ary operations of the algebraic theory* $U\hat{\mathfrak{S}}$ *are the natural transformations* $U^n \to U$.

Thus, any category $\mathfrak{X}$ equipped with an "underlying set functor" U determines an algebraic category $\mathcal{S}^{(U\hat{\mathfrak{S}})}$ together with a functor $\Phi:\mathfrak{X} \to \mathcal{S}^{(U\hat{\mathfrak{S}})}$ which preserves underlying sets, and given any other such functor $\Psi:\mathfrak{X} \to \mathcal{S}^{(\mathbf{A})}$, there is a unique $f:\mathbf{A} \to U\hat{\mathfrak{S}}$ in $\mathfrak{I}$ such that $\Psi = \Phi\mathcal{S}^{(f)}$. Also, the operations which define an algebraic category are in natural one-to-one correspondence with the natural operations on its underlying set functor. Thus the functor $\hat{\mathfrak{S}}$ becomes most interesting when applied to "underlying set functors" on nonalgebraic categories. For example, if we take for $\mathfrak{X}$ the dual of the category of sets and for U the (contravariant) power set functor, the algebraic structure of U is the theory of Boolean algebras and Φ assigns to each set the Boolean algebra of its subsets. Again, if we take U as the functor which assigns to every group G the set of all integer-valued functions on G with finite support, and to every group homomorphism $g:G \to G'$ the function $gU:GU \to G'U$ defined by $(x')(f)(gU) = \Sigma\{xf|xg = x'\}$ for $f \in GU$, $x' \in G'$, then $U\hat{\mathfrak{S}}$ is an extension of the theory of rings which includes two additional unary operations, "involution" and "trace."

The above theorem implies that a category $\mathfrak{X}$ is equivalent to some algebraic category *iff* it has some underlying set functor U such that the particular functor $\Phi:\mathfrak{X} \to \mathcal{S}^{(\mathbf{A})}$ described above is an equivalence where $\mathbf{A} = U\hat{\mathfrak{S}}$. In that case we must actually have $U = \mathrm{Hom}(G, ?)$ where G is an object in $\mathfrak{X}$ such that $G\Phi$ is a free $\mathbf{A}$-algebra on one generator. These observations enable us to completely characterize algebraic categories in the theorem below. We first define some terms.

By an *equalizer* and *coequalizer* of a pair of maps

$$X \overset{f_0}{\underset{f_1}{\rightrightarrows}} Y$$

in a category $\mathfrak{X}$, we mean respectively an inverse limit $j:K \to X$ and a direct limit $p:Y \to K^*$ of the above diagram in the sense of Kan.[3] A map p is called a *regular epimap iff* it is a coequalizer of some pair of maps. A category *has finite limits iff* it has an initial and a final object and if all possible binary products and coproducts, as well as all possible equalizers and coequalizers, exist. A pair f_0, f_1 as above is called a *precongruence iff* the corresponding $f:X \to Y \times Y$ is monic as well as reflexive, symmetric, and transitive in an obvious sense; the pair is called a *congruence iff* f is the equalizer of the pair $<\pi_0 p, \pi_1 p>$ where $\pi_i:Y \times Y \to Y$ are the projections and p is the coequalizer of $<f_0, f_1>$. In a category with finite limits, every congruence is a precongruence, while in any algebraic category the converse is also true.

An object G in a category $\mathfrak{X}$ is *abstractly finite iff* the following three conditions hold: (*a*) for every object X, the class $\mathrm{Hom}(G, X)$ of maps $G \to X$ is small (i.e., is a "set"); (*b*) for every small class I, the I-fold coproduct (= free product) $I \cdot G$ exists; (*c*) any map $G \to I \cdot G$ factors through some $I' \cdot G$ where $I' \subset I$ and I' is finite. A map $f:Y \to Z$ in $\mathfrak{X}$ is *G-surjective iff* every map $G \to Z$ factors across f. An object G is a *regular projective generator iff* the G-surjective maps are precisely the regular epimaps.

THEOREM. *Let $\mathfrak{X}$ be a category with the following properties: (0) $\mathfrak{X}$ has finite limits, (1) $\mathfrak{X}$ has an abstractly finite regular projective generator G. Then there is an algebraic theory $\mathbf{A}$ and a functor $\Phi:\mathfrak{X} \to \mathcal{S}^{(\mathbf{A})}$ which is full, faithful, and has an adjoint, and the free objects in $\mathfrak{X}$ coincide with those in $\mathcal{S}^{(\mathbf{A})}$ (i.e., $(I \cdot G)\Phi = I \cdot (G\Phi)$). Furthermore, Φ is an equivalence iff (2) every precongruence in $\mathfrak{X}$ is a congruence. Conditions 0, 1, 2 are necessary and sufficient that $\mathfrak{X}$ be equivalent to some algebraic category.*

This theorem is closely related to an unpublished theorem characterizing "quasi-primitive" categories of algebras which was found by J. R. Isbell, who also pointed out that my original proof of the above could be simplified by noting that under conditions 0 and 1, every subalgebra of $X\Phi$, for X in $\mathfrak{X}$, is itself of the form $X'\Phi$. A category satisfying 0 and 1, but not 2, is that of all torsion-free abelian groups; the adjoint to Φ in this case consists of dividing by the torsion subgroup.

COROLLARY. *If $\mathfrak{X}$ is an algebraic category and if $\mathcal{C}$ is any small category with finitely many objects, then the full category $\mathfrak{X}^{\mathcal{C}}$ of functors $\mathcal{C} \to \mathfrak{X}$ and natural transformations thereof is itself equivalent to an algebraic category.*

The proof of this corollary involves interpreting the underlying set of a functor to mean the product of the underlying sets of the objects involved. When $\mathcal{C}$ has exactly one object, then $\mathcal{C}$ is a monoid and the corollary refers to the well-known notion of a monoid acting by endomorphisms of an algebra to form a new algebra.

COROLLARY. *An abelian category is algebraic iff it is the category of all modules over some associative ring with unity.*

This follows immediately from a theorem of Freyd;[2] condition 2 of the theorem above is always satisfied in an abelian category. The nature of the rings whose existence is implied by these two corollaries is being investigated.[4]

* This constitutes a partial summary of a dissertation submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy at Columbia University. The author is grateful to Professors Eilenberg, Mac Lane, and Freyd for their inspiration and encouragement.

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*THE CRYSTAL STRUCTURE OF AN INTERMOLECULAR NUCLEOSIDE COMPLEX: ADENOSINE AND 5-BROMOURIDINE**

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The concept of hydrogen-bonding specificity between the purine and pyrimidine bases, adenine and thymine, guanine and cytosine, is fundamental in the present theory of nucleic acid structure and replication. The base-pairing scheme proposed by Watson and Crick[1] in their structural hypothesis for the DNA molecule has gained wide acceptance among biologists and recently has received strong support from three-dimensional Fourier analysis of DNA fiber X-ray diffraction data,[2] and from structure analysis of single crystals containing guanine and cytosine derivatives in an intermolecular complex.[3, 4] On the other hand, Hoogsteen[5] has found a different base-pairing configuration in a crystalline complex of 9-methyl adenine and 1-methyl thymine. Here, the ring nitrogen N3 of thymine hydrogen-bonds to the imidazole nitrogen N7 of adenine instead of bonding to N1 as in the Watson-Crick model. A similar pairing configuration has recently been found in a crystalline complex containing 9-ethyl adenine and 1-methyl uracil.[6] This pairing is of considerable interest since it is thought to occur in the triple-stranded 2:1 complex of polyuridylic acid and polyadenylic acid.[7]

The information derived from these structure investigations has prompted us to investigate other possibilities for cocrystallization of important compounds known to interact in biological systems. The present work describes a single crystal analysis of a nucleoside intermolecular complex between adenosine and 5-bromouridine. The presence of the sugars on the purine and pyrimidine bases brings this model system close to the biological systems of interest. Furthermore, the bromine-substituted derivative is of particular interest since the closely related molecule bromodeoxyuridine is a well-known mutagenic agent. The results show the existence of a third type of base-pair configuration.

Methods.—The adenosine-5-bromouridine complex was crystallized in clusters of thin needles by slow evaporation from an aqueous solution containing equimolar quantities of these compounds. Ultraviolet absorption measurements on aqueous solutions made from single crystals confirmed the presence of the two nucleosides in approximately equal proportions. The crystals were found to be orthorhombic, space group $P22_12_1$ with $a = 4.80 \pm 0.01$, $b = 15.19 \pm 0.01$, and $c = 31.76 \pm 0.03$ Å; the density determined in benzene-methyliodide solutions was 1.706 ± 0.010 gm/cc. The unit cell contains four asymmetric units, each consisting of an adenosine-bromouridine pair and a water molecule. Equi-inclination Weissenberg photographs were taken about the a axis with filtered Cu$K\alpha$ radiation, using the multiple film technique. The intensities were estimated visually and corrected with the appropriate Lorentz-polarization factors. A total of 2,511 reflections were indexed, of which 2,015 were nonzero, representing about 90 per cent of the data accessible in the copper sphere. No correction was made for absorption effects.

Determination of the structure: The bromine atom position was determined from the three-dimensional Patterson function. The heavy atom method was first applied to the centrosymmetric (0kl) projection yielding a trial structure for the bromouridine molecule. Successive Fourier syntheses revealed the complete two-dimensional structure, as shown in Figure 1. A three-dimensional Fourier based on the bromine phases was then easily interpretable, and most of the atomic positions could be determined unambiguously. A second three-dimensional Fourier based on this structure established the complete structure of the nucleoside pair and revealed an extra peak of low electron density. This was tentatively identified as a water molecule.

The structure was refined by the method of least squares using a modified version of the full matrix least squares program for the IBM 7090 computer devised by Busing, Martin, and Levy.[8] The presence of a water molecule associated with the asymmetric unit was confirmed during the course of refinement; variation of the atom multiplier led to a final value of 0.6 water molecules per nucleoside pair, in good agreement with the value of 0.4 obtained from the crystal density. Successive cycles of individual atom isotropic refinement has reduced the over-all residual factor to 12 per cent. The standard deviation for light atom bond lengths is ±0.03 Å based on the standard errors of atomic coordinates calculated from the full normal matrix.[8]

Description of the Structure.—Figure 2 shows the crystal structure viewed down the *a* axis. Presumed hydrogen-bonding contacts are indicated by dashed lines, and the distances are given. Dotted lines indicate other distances of interest. The existence of a strong hydrogen bond between the bases is clearly indicated between N3 of bromouridine and N7 of adenosine with a length of 2.80 Å. A second weak hydrogen bond appears to occur between O2 of bromouridine and N6 of adenosine. The C6-N6-O2 angle of 126° is favorable for hydrogen bonding; however, the distance of 3.10 Å is too long. It is possible that the bond is strained by the interactions of the bromouridine O4 and adenosine O5′ with an adjacent sugar or that the proximity of the water, 2.90Å from O2 of uracil and 2.99 Å from N6 of adenine, perturbs the base pairing.

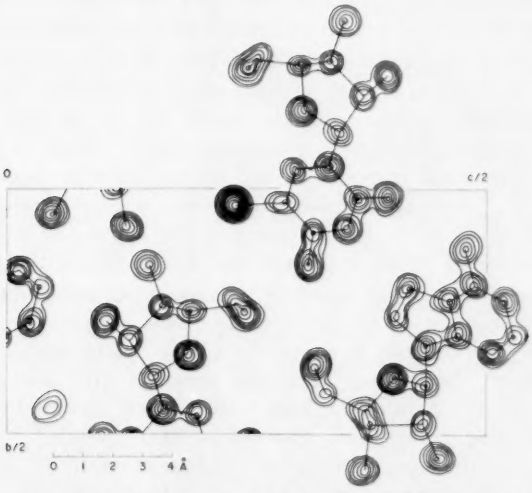

FIG. 1.—(0kl) Fourier projection of the adenosine-5-bromouridine crystal structure. Contours are drawn at equal but arbitrary levels of electron density. The bromine atom contours are drawn for every fourth level of electron density. The extra peak shown in the lower left-hand corner is a water molecule; its low level of electron density is attributed to incomplete occupancy in the crystal lattice.

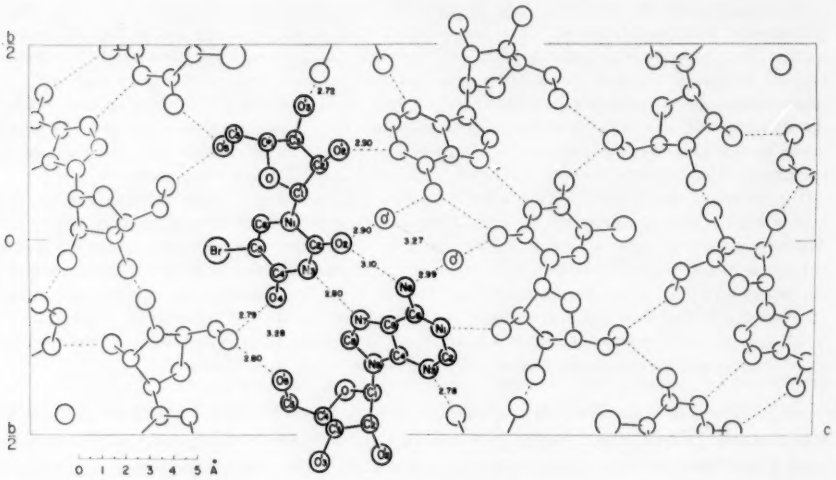

FIG. 2.—Packing diagram of the adenosine-5-bromouridine crystal structure viewed down the *a* axis. Presumed hydrogen-bond contacts are drawn with dashed lines and the distances shown. Dotted lines indicate other contacts of interest.

All hydroxyl groups of the two ribose rings are involved in hydrogen bonding with each other and with the remaining electron donors of the two bases, N1 and N3 of adenine, and O4 of uracil. These bonds are important in maintaining the integrity of the crystal structure along the *b* and *c* axes. The O—H· · ·O distances observed are 2.72, 2.79, and 2.80 Å; the O—H· · ·N distances are 2.78 and 2.90 Å. The bromine atom does not appear to participate in hydrogen bonding; the closest approach is 3.61 Å from C5′ and O5′ of a neighboring adenosine molecule.

Covalent bond distances and angles have been computed and are in good agreement with those found in crystals of adenosine-5′-phosphate,[9] calcium thymidylate,[10] and fluorodeoxyuridine.[11] These results will be reported in detail elsewhere. Least squares planes were determined for the atoms of the bromouracil ring, including the sugar carbon C1′, and for the adenine ring system with its sugar carbon C1′. Both gave excellent fits to least squares planes with maximum deviations of 0.025 Å in the former case and 0.035 Å in the latter with the exception of adenosine C1′ which deviates by 0.05 Å from the adenine plane.

The two ring systems were found to be almost, but not completely, coplanar, with a dihedral angle of 4° 55′ between their least squares planes. The amino nitrogen of adenine lies 0.03 Å above the adenine plane and 0.04 Å below the bromouracil plane. Both ribose sugars are puckered with carbon C3′, deviating by approximately 0.6 Å from the least squares plane formed by the other four members of the ring, as has been observed in other crystals.[9, 10] The angle between the plane of the sugar and that of the base is about 70° in each case; the distance between the glycosidic carbons C1′ of the two nucleosides is 9.65 Å.

The water molecule associated with the asymmetric unit is located near the twofold rotation axis and is indicated by O′ in Figure 2. It appears to interact weakly with O2 of uracil and N6 of adenine, as indicated by the dashed lines. With

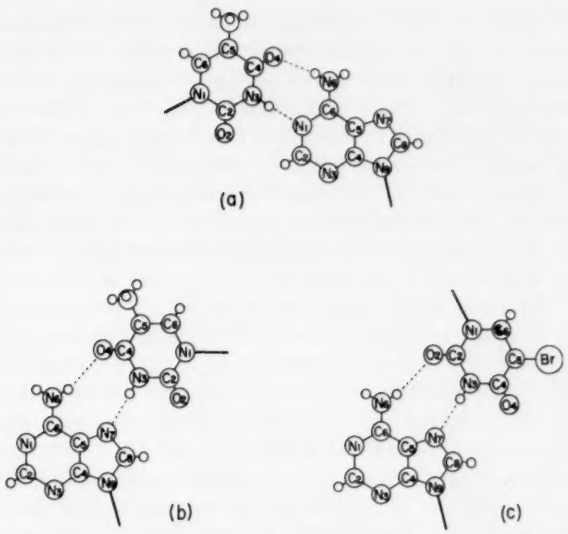

FIG. 3.—Schematic diagram of various base pairings between adenine and thymine. (*a*) The Watson-Crick pairing hypothesized for DNA. (*b*) Pairing found by Hoogsteen between 9-methyl adenine and 1-methyl thymine. (*c*) Pairing found in the present structure determination of the adenosine-5-bromouridine complex.

respect to the bases with which it makes closest contact, it lies 0.07 Å above the adenine plane and 0.24 Å above the bromouracil plane.

Discussion.—An important feature observed in the crystal complexes of nucleic acid components studied thus far is the hydrogen bonding between the purine and pyrimidine bases with resultant formation of a highly planar complex.[3–6] This is illustrated convincingly in the present structure, as shown in Figure 2. Here, in spite of the complexity of the over-all crystal structure, the base pair itself is almost planar, having a dihedral angle of 4°55′ between the least squares planes of the individual bases. It is for this reason that we consider the hydrogen-bonded base pair, as observed in the crystal structure, to be an intermolecular complex, which may exist in solution prior to crystallization. Purine and pyrimidine compounds are found to associate extensively in aqueous solutions, although the actual mechanism is unknown.[12]

Figure 3 shows the base pairing arrangement believed to exist in DNA,[1] the pairing found by Hoogsteen,[5] and that found in the present investigation. In both the Watson-Crick and the Hoogsteen pairings the carbonyl oxygen O4 of thymine hydrogen-bonds to the amino group N6 of adenine. In the adenosine-bromouridine crystal complex, however, the amino group of adenine appears to hydrogen-bond to the carbonyl oxygen O2 of bromouracil, although the distance 3.10 Å is rather long, indicating that interaction between these groups is weak. The carbonyl oxygen O4 hydrogen-bonds to a neighboring sugar hydroxyl group.

We are particularly interested in the difference in hydrogen bonding of the carbonyl oxygens of uracil in our crystal compared with that in the Watson-Crick and the Hoogsteen structures. Is this the result of crystal packing forces and

sugar hydroxyl group hydrogen bonding, or does it reflect an alteration in the physical properties of the uracil ring due to the presence of the bromine atom? Another important question is whether or not the base-pair configuration found in the crystal structure truly reflects interactions which exist in solution. If this is so, then the possible influence of the bromine atom should be considered.

The effect of halogen substitution in fully conjugated systems has been studied extensively.[13] Observations on the chemical and physical properties of chlorobenzene, for example, have indicated the importance of resonance effects involving the contribution of a p electron from the halogen atom to the π electron system of the ring. This would tend to increase electronegativity at the ortho- and para-positions. On the other hand, inductive effects, which become more important in systems only partially conjugated, act in the opposite direction. In the case of uracil one might expect the bromine atom to exert primarily an inductive effect on the ring; this would tend to decrease electronegativity of the ortho-carbonyl O4 relative to the para-carbonyl O2. This may explain why the amino group of adenine bonds to O2 rather than to O4 of 5-bromouracil.

A powerful control study, on a complex of adenosine and thymidine, has not been possible as yet, because we have been unable to cocrystallize these compounds. Comparison of a complex of 9-ethyl adenine and 1-methyl-5-bromouracil currently under investigation[14] with the Hoogsteen crystal should be valuable; preliminary results indicate the possibility of the same base-pairing as in adenosine-bromouridine. We are inclined to be cautious, however, about making direct comparison of the nucleoside complexes with these systems, as well as with DNA, because of the great differences in their properties in solution in addition to the crystal-packing factors. For example, hydrophobic interactions may play a larger role in determining the base-pair configuration in solution in a system like the Hoogsteen complex, possibly masking effects that are important in nucleoside complexes. The actual occurrence of a water molecule in the adenosine-bromouridine crystal structure and its location provide additional reason to be concerned about the possible role of water in the hydrogen bonding between the bases.

It is of considerable interest that the present structure shows a strong hydrogen bond between N3 of bromouracil and N7 of adenine. The closeness of the contact (2.80 Å) compared with the usual N—H$\cdots$N bond distance of about 2.95 Å[13] suggests that other forces between the bases such as dipole-dipole interactions may be important. This bond also occurs in the adenine-thymine and the adenine-uracil complexes previously studied. It is not clear why it should be favored over the Watson-Crick bonding between uracil N3 and adenine N1. In the present crystal structure N1 is observed to interact with an adjacent sugar hydroxyl group, although in the other reported crystal complexes it shows no hydrogen bonding.

Another interesting feature of the adenosine-bromouridine structure is the presence of a water molecule near the carbonyl oxygen O2 of uracil and the amino nitrogen N6 of adenine. Least squares refinement of the structure has established that statistically there is only 0.6 of a water molecule at this site, indicating incomplete occupancy in the crystal lattice. This is not entirely surprising considering the lack of strong hydrogen bonding by the water molecule. However, the presence of the water molecule, together with the effects of hydrogen bonding by the sugar

hydroxyl groups, may be important in causing distortion of the base-pair configuration and lengthening of the O2-N6 hydrogen-bonding contact.

The results of this structure determination have shown the existence of a different type of base pairing between the adenine and uracil rings in the nucleosides. Whether or not this pairing is in any way related to the phenomenon of mutation is an intriguing question. Preliminary attempts at model building have indicated that it would be difficult, if not impossible, to incorporate this base-pairing configuration into the DNA molecule as it is currently conceived. Further work is proceeding on other nucleoside crystal complexes, with particular attention to their hydrogen-bonding arrangement, the degree of planarity of the base pairs, and the relation of the sugar residues to the bases. Crystal complexes of deoxyguanosine with 5-bromodeoxycytidine[15] and inosine with 5-bromodeoxycytidine[16] are currently being investigated. The former shows the Watson-Crick pairing with the bases approximately coplanar and joined by three hydrogen bonds.

Summary.—The crystal structure of an intermolecular complex containing adenosine and 5-bromouridine has been presented. The results demonstrate the existence of a third type of base pairing which is different from that occurring in DNA as hypothesized by Watson and Crick and that found by Hoogsteen in a crystal complex of 9-methyl adenine and 1-methyl thymine. The significance and possible biological implications of this new structure are discussed briefly.

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‡ Helen Hay Whitney postdoctoral fellow.

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*ANIMAL AND PLANT VIRUSES WITH DOUBLE-HELICAL RNA**

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THE ROCKEFELLER INSTITUTE

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Reoviruses[1, 2] possess outstanding physical and biological properties. The viral genetic material is an extraordinarily stable double-helical RNA[3, 4] of an estimated molecular weight in excess of 10×10^6. The viruses are more widely distributed among animal species, including man, than any other virus known.[5–7] Antibodies to reoviruses have been found in all vertebrates, except whales.[6] Reovirus is a virus in search of disease; no definite association between the virus and disease has been found in man, monkeys, cattle, or dogs.[5–7]

That a plant virus, the wound tumor virus, shares a number of properties with reovirus has aroused speculations concerning the relationship between the two.[3, 8] The plant virus, which multiplies and causes disease in a great variety of plants and tumors in some,[9] is similar to reovirus in size and surface structure. The wound tumor virus and reovirus both measure approximately 700 A in diameter and the capsid of each possesses 92 morphological units.[5, 10–13] In their interaction with cells, both wound tumor virus and reovirus produce characteristic cytoplasmic inclusion bodies which contain virus particles.[5, 14–16] There is no structural evidence of nuclear involvement in the growth of either virus.

Wound tumor virus was first isolated from leafhoppers in which host it also multiplies.[10] The leafhoppers can transmit the virus to more than 43 species of plants distributed in 20 different plant families, including sweet clover and crimson clover, chrysanthemums, and sorrel.[9] Virus isolated from plants and insects is similar in size, structure, sedimentation behavior, and antigenic constitution.[10]

The structural similarities between wound tumor virus and reoviruses suggested that the nucleic acid of wound tumor virus might also be a double-stranded polyribonucleotide, and our preliminary results indicated that this is indeed the case.[3] We have now obtained additional evidence which supports this conclusion. Furthermore, we have found that wound tumor virus RNA contains less G + C and has a lower melting temperature than reovirus RNA.

One of the intriguing biological questions is whether reoviruses—alone or in conjunction with physical or chemical agents—cause tumors in man and animals. There is no answer to this question at present; however, we have been stimulated to consider what the fundamental and necessary properties of the genetic material of tumor viruses might be. We propose that double-strandedness of the nucleic acid, assuring stability of the genetic information, may be one such property.

Materials and Methods.—Cuttings from clone C10 of infected sweet clover were kindly made available by Dr. L. M. Black. Clover plants were grown in soil in a greenhouse whose temperature varied between 75 and 80°. The light was on for 12–14 hr each day. After 2 months, since only a few small tumors were visible on the plant stems, the stems were injured in numerous places with a pin. One month later, numerous tumors had formed and were removed from the stem with a knife. Three to 4 gm of tumor material were removed on each of two occasions from the stems, and 500 gm from the roots on one occasion; all the material was

frozen at −28°. The tumors were washed with running water and then ground with alundum in phosphate-buffered saline (PBS) to a thick paste. The homogenate was diluted with PBS to give a 10% suspension, filtered through fine gauze, and then centrifuged at 10,000 *g*. The supernate which contained the virus was subjected to the same purification procedure described previously for reovirus,[3] including centrifugation, enzymatic and fluorocarbon treatments, and banding in a cesium chloride density gradient. The main band had an average density of 1.411. A preparation which had not been treated with enzymes or fluorocarbon, but had been banded in the cesium chloride gradient, was examined by Dr. Samuel Dales in the electron microscope and was found to contain characteristic virus particles and minimal amounts of cellular contaminating material.

Results.—Base composition of wound tumor virus RNA: The nucleic acid was extracted from the virus particles with phenol at room temperature.[17] The maximum absorbancy of the virus RNA was at 261 and the minimum at 235 mμ. The RNA was analyzed by the procedure of Smith and Markham[18] to determine its base composition. As shown in Table 1, the mole per cent of guanine closely

TABLE 1

BASE COMPOSITION OF WOUND TUMOR VIRUS AND REOVIRUS RNA

Sample	Wound Tumor Virus RNA Bases (mole %) G	A	C	U	G + C
A	18.4	30.2	19.3	32.2	37.7
B	18.8	31.9	18.9	30.4	37.7
Mean	18.6	31.1	19.1	31.3	37.7
		Reovirus type 3 RNA			
Mean value*	22.3	28.0	22.0	27.9	44.3

* Based on 5 analyses on 4 preparations.

approximates that of cytosine, and the mole per cent of adenine approximates that of uracil. The mole per cent of G + C is 38 per cent of total. Thus far, we have had available enough wound tumor virus RNA for only two analyses on samples obtained from the same starting material but purified separately. The results on the two samples agreed closely, as shown in Table 1.

Further results on the base composition of five samples of reovirus type 3 have resulted in a refinement of the G + C value. The new mean value of 44 per cent is similar to the value of 42.7 per cent reported previously on the first preparation examined. For reasons unknown, the second of the two preparations examined previously gave a much lower G + C content (36.8%) than all other preparations of reovirus which have been examined. Thus, the G + C content of wound tumor virus RNA appears to be significantly lower than that of reovirus RNA.

Thermal denaturation of wound tumor virus RNA: Figure 1 shows that, on heating to 95° in a medium of 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.0, wound tumor virus RNA exhibited a hyperchromic effect of 27 per cent. Absorbance began to increase at 87°, and the T_m of 90° was similar to that reported previously.[3] A third preparation of wound tumor virus RNA melted at a slightly higher temperature, namely 92°. The total increase in absorbancy observed in different experiments has varied between 27 and 36 per cent.

In two experiments with wound tumor virus RNA, parallel observations were made on reovirus RNA in the same sodium chloride-citrate medium. Reovirus

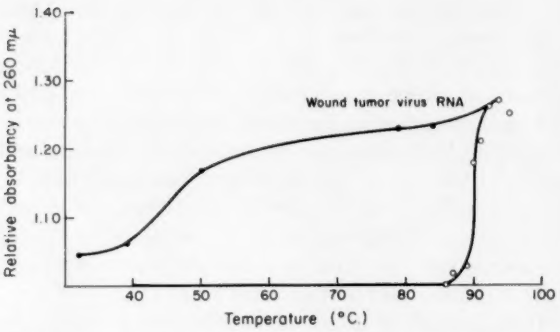

FIG. 1.—Thermal denaturation of wound tumor virus RNA. Wound tumor virus RNA in 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.0 was heated in a quartz cuvette in a thermostatically controlled Beckman spectrophotometer, and absorbancy at 260 mμ was determined at various temperatures (O—O). After denaturation was complete, the sample was allowed to cool slowly to 31° over a 2-hr period (•—•).

RNA began to melt at a temperature about 3° higher than wound tumor virus RNA. On slow cooling, both the wound tumor virus RNA and the partially melted reovirus RNA regained almost completely their original hypochromicity. In the sodium chloride-citrate medium the T_m of reovirus RNA[3] is approximately 3° higher than that of wound tumor virus RNA.

Lack of immunological relationship between wound tumor virus and reoviruses: With the obvious similarity in the secondary structures of the genetic material of these two viruses, experiments were performed to determine if there were also immunological similarities in their protein coat in addition to the reported similarity in capsomeric number. Immune sera were prepared by intravenous injection of the viruses two times into rabbits at two-week intervals; the sera were collected two weeks after the second injection. Purified wound tumor virus and a lysate of reovirus-infected L cells were used as antigens. The sera were inactivated by heating at 56° for 30 min. Complement fixation was performed by the method recommended by the Viral and Rickettsial Disease Laboratory of the California State Department of Public Health. The sera were further treated with *V. cholerae* filtrate overnight at 37° prior to testing their inhibitory activity on viral hemagglutination and plaque formation.

As can be seen in Table 2, despite the strong fixation of complement by all three prototype strains of reovirus in the presence of immune reovirus 3 serum, there was no cross reaction when wound tumor virus was used as the antigen. Immune serum

TABLE 2

LACK OF IMMUNOLOGICAL RELATIONSHIP BETWEEN WOUND TUMOR VIRUS AND REOVIRUSES

	Complement Fixation Titer			
Antigen	Preimmune serum	Wound tumor virus immune serum	Preimmune serum	Reovirus 3 immune serum
Wound tumor virus	16	64	16	8
Reovirus 1 (Lang)	8	8	<8	>256
Reovirus 2 (Amy)	8	8	<8	128
Reovirus 3 (Dearing)	8	<8	<8	>256

against wound tumor virus had a complement fixation titer of 64 with tumor virus as the antigen; the titer of the preimmune serum was 16. There was also no evidence of immunological cross relationship when the three prototype strains of reovirus and wound tumor virus immune serum were reacted. Furthermore, hemagglutination inhibition and neutralization tests gave no evidence of antibodies against any of the three prototype strains of reovirus in wound tumor virus immune serum.

Thus, we have found no evidence of an immunologic relationship between any of the three types of reovirus and wound tumor virus. While this manuscript was in preparation, a report appeared by Streissle and Maramorosch[8] that there did exist a common complement-fixing antigen in wound tumor and reoviruses. The results which were presented are, however, not convincing. The titers of reovirus preparations employed as antigen in complement fixation tests were unusually low. In addition, to evaluate the significance of complement fixation titers obtained with reoviruses and wound tumor virus hyperimmune serum, the titers were compared with those obtained with pooled normal rabbit serum, rather than preimmune serum from the rabbits from which immune serum was obtained. Because of the ubiquity of reoviruses and their ability to infect rabbits, results of complement fixation lose some of their significance when the preimmune and immune sera are not from the same animal.

Experiments on interaction of wound tumor virus with mammalian cells: Wound tumor virus, purified as described above and dialyzed against phosphate-buffered saline, did not agglutinate human, bovine, guinea pig, or rat erythrocytes either at room temperature or at 4°, nor did it agglutinate chick erythrocytes. Inoculation of the virus suspension onto monolayers of monkey kidney cells or mouse fibroblasts (L929) and passage serially three times over a 41-day period produced no evident cytopathic effects on the monolayers. Subcutaneous and intranasal inoculation of 24-hour-old mice and hamsters with wound tumor virus produced no overt illness during $5^1/_2$ months of observation.

Discussion.—We have obtained evidence that the ribonucleic acids of wound tumor virus and of reovirus have a highly ordered secondary structure, namely, a double-stranded helix. The amount of nucleic acid per reovirus particle is greater than 10×10^6. It is probable, from the volume available to the nucleic acid in the virus particle,[11] that the amount of RNA in wound tumor virus is similarly large. Reo- and wound tumor viruses thus differ from many RNA viruses, all of which possess a complement of about 2×10^6 molecular weight units of single-stranded RNA.[19, 20] Polio- and influenza viruses represent well-studied examples of such cytocidal RNA viruses, whose growth is characterized by a short latent period, followed by a rapid increase in virus. It is of great interest that in contrast to these agents, two tumor viruses of birds contain RNA in an amount considerably larger than the usual 2×10^6 molecular weight units.[19, 21, 22] Whether the RNA of these avian tumor viruses, Rous sarcoma and avian myeloblastosis, is double- or single-stranded, remains to be determined.

It has been suggested that viruses which possess a number of common properties may be considered as related, and that such a conclusion is valid apart from considerations of evolutionary relatedness.[23] On this basis reo- and wound tumor viruses, although they affect widely different hosts, are related agents. It has been

reported that these two viruses have a common complement-fixing antigen.[8] If this were so, evolutionary relatedness would appear likely. However, in the present study we found no evidence of any serological relationship between wound tumor virus and any of the three immunological types of reovirus. Moreover, wound tumor virus failed to agglutinate a variety of erythrocytes and to cause cytopathic effects in mammalian cells. It is not surprising that the base compositions of these two viruses are different. The mole per cent of G + C in wound tumor virus RNA is 38, whereas in reovirus it is 44. Furthermore, the melting temperature of wound tumor virus RNA is lower than that of reovirus RNA, probably reflecting the lower G + C content of the former.

There is a third virus which shares structural and biological characteristics with reo- and wound tumor viruses. The rice dwarf virus produces a dwarfing disease in rice plants and multiplies both in the plant and in its insect vector,[24] again the leafhopper. The size of the rice dwarf virus particles is 750 A and the number of structural subunits may be similar to that previously described for reo- and wound tumor viruses, i.e., 92. Like reo- and wound tumor viruses, the rice dwarf virus appears to multiply only in the cytoplasm of the infected cell. Virus particles form crystalline arrays and inclusion bodies are seen. The wound tumor virus and the rice dwarf virus are two of the few viruses known to bridge the plant and animal kingdoms.[25] The pathological changes produced by the viruses in their various hosts are different; indeed, the wound tumor virus does not adversely affect its insect vector,[26] whereas the rice dwarf virus does.[24] If it is shown that the nucleic acid of rice dwarf virus is also a double-helical polyribonucleotide, this would provide additional evidence that this relatively large and unusual structure may be endowed with special characteristics which permit replication in widely different hosts.

The fact that wound tumor virus is capable of causing tumors raises the question whether reovirus may also be oncogenic. The answer is not yet available, and the problem may be complex. Although the wound tumor disease in plants is induced by a virus, the development of tumors in a host is largely limited to areas of irritation.[26, 27] As emphasized by Braun,[27] a host may be systemically infected with the virus and yet no tumor is produced unless cells are triggered to divide in response to injury. Furthermore, the genetic constitution of the host plays an important role in the expression of this disease.[28] These observations quite obviously provide guidelines for investigation of the possible tumorigenic potentialities of reoviruses. A variety of physical and chemical agents, including hormones, will have to be considered as possibly important supplemental factors.[29]

The results which we have obtained so far do bear on the important problem of fundamental properties which characterize the genetic material of tumor viruses. On the basis of what is known about the biology of virus-induced tumors, it may be postulated that tumor viruses have at least two fundamental characteristics. First, their nucleic acid must have the required stability to assure the presence in the infected cells of viral genetic material for a considerable period of time. Continued presence of virus-specific elements has been demonstrated in all virus-induced tumors save polyoma, in which case all attempts to prove their continued presence have failed.[30–34] Second, the nucleic acid of tumor viruses must be able to replicate and to express its gene functions in ways which will allow synthesis of

host cell materials to continue, and, in addition, tumor virus genetic material must be potentially capable of subverting the cell from the path of normal growth and division to uncontrolled growth and division.[27, 34]

The DNA of tumor-producing viruses has been shown to be double-stranded in all cases,[35–39] and thus stable to thermal denaturation. The double-stranded helical configuration not only offers stability to denaturating agents, but also allows the intercellular transfer in one molecule of a relatively large amount of genetic information which may be a necessary requirement for tumor induction.

That viruses with single-stranded RNA have not been shown to be able to cause neoplastic transformation of cells may be highly significant. There is evidence that neither RNA nor DNA, when free and single-stranded, possesses the necessary stability to function as a repository of information for a prolonged period at 37°. The molecular half-life, that is, the time for the molecular weight of a polynucleotide of 10,000 units to be halved, was found to be 15 hr for single-stranded RNA, and 10 days for single-stranded DNA.[40] No values were given for double-stranded DNA, but presumably this configuration is much more stable. The mutability of small RNA viruses and the relative stability of DNA viruses are well known. As we have shown, the double-stranded RNA of reo- and wound tumor viruses possesses extraordinary stability to heat.

In addition to the double-stranded helical configuration, the nucleic acids of the DNA-containing tumor viruses possess an additional stabilizing characteristic manifested by the remarkable renaturation behavior of these molecules. When these nucleic acids have been denatured by heat, they have either retained full infectivity in the instances where an assay was available, or they have regained partially or completely their original hypochromicity or their original density in cesium chloride or sulfate.[31, 35, 36, 41–43] Other DNA's acquire similar reannealing capacity through treatment with ultraviolet light, X-rays, or nitrogen mustards—all tumor-inciting principles.[44, 45] The RNA's of wound tumor virus and reovirus regain their original hypochromicity upon slow cooling from 95°C. Thus, the genetic material of both viruses has the stability which we would expect in tumor viruses, and wound tumor virus is in fact tumorigenic in a susceptible host, the sweet clover plant.

Reovirus types 1 and 2 have not been demonstrated to cause any overt illness in nature; only specially adapted strains have produced illness in mice in the laboratory.[6] Reovirus 3, when inoculated in large amounts into newborn laboratory mice or hamsters, can cause an hepatoencephalomyelitis. However, animals only a few days old are no longer susceptible. It is not known in which cells of the numerous and various hosts this group of viruses preferentially multiplies and what the inapparent cytopathology may be. Under artificial conditions in tissue culture, when mouse fibroblasts are inoculated with a large amount of reovirus type 3, the effects of reovirus infection on cellular macromolecular syntheses and association of reovirus with certain cellular structures are known. Reovirus, unlike several of the cytocidal RNA viruses, does not grossly affect the synthesis of RNA and proteins in infected cells, but does inhibit DNA metabolism,[46] suggesting a specific effect on the genetic apparatus of the cell. Furthermore, in reovirus-infected cells undergoing mitosis, virus seems to be located in a preferred but not necessary association with the mitotic spindle of the host cells.[47, 48] It is possible that the

virus on the spindle could interfere with distribution of cellular genetic material during division.

Reoviruses thus have some of the necessary attributes of tumor viruses. Only further experiments can show whether they are in fact capable of causing tumors.

Summary.—The genetic material of wound tumor virus, like that of reovirus, is a large double-helical ribonucleic acid. In wound tumor virus RNA the mole per cent of guanine is 19, adenine 31, cytosine 19, and uracil 31. The melting temperature of 90°, about 3° lower than that of reovirus RNA in the same medium, probably reflects the lower G + C content in wound tumor virus RNA, namely 38 versus 44 per cent. There is no serological relationship between wound tumor virus and any of the three prototype strains of reovirus. Reoviruses possess some of the necessary attributes of viruses with tumorigenic capacity.

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*EVOLUTION OF HEMOGLOBIN IN PRIMATES**†

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Several proposals have been made to describe the evolutionary relationships among the genes that control the structure of the polypeptide chains in the normal human hemoglobins.[1-3] At present, the best means for evaluating these proposals is by analysis of the sequence of amino acids in the polypeptide chains of hemoglobins from several vertebrate and invertebrate forms. With this information, homologies of chain structure as shown by correspondence between sequences of amino acids in entire chains, or portions of chains, help to establish the order or trend of evolution of the hemoglobin genes. Because a vast number of animals are available for this purpose, it is important that a careful choice of subjects for study be made. The principal basis for such choice should be the comparative anatomy and paleontological record of the animal groups. Thus, one will study hemoglobins from those animals that, in contemporary theory, represent stages in the development from primitive to more advanced forms, and finally to man. For this reason we have chosen to study the hemoglobins from a single mammalian order, the Primates. This order contains not only man, of whose hemoglobins so

much is known, but also representatives, still living, that reflect almost all grades of primate evolution from the most primitive to the most advanced forms.[4]

We report here preliminary results of studies on the structural analysis of hemoglobins from several primates. From this information, it is possible to examine the evolutionary relationships of hemoglobins among primates.

Materials.—Hemoglobins from the following animals have been examined: *Hylobates lar* (gibbon), *Macaca mulatta* (macaque), *Cercopithecus mitis* (Sykes monkey), *Papio doguera* (baboon), *Cacajao rubicundus* (uakari), *Saimiri sciurea* (squirrel monkey), *Galago crassicaudatus* (bush baby), *Perodicticus potto* (potto), *Propithecus verreauxi* (Sifaka), *Lemur catta* (ring-tailed lemur), *Lemur fulvus* (brown lemur), *Lemur variegatus* (ruffed lemur), and *Tupaia glis* (Malayan tree shrew). Blood samples were collected and hemolysates prepared by methods described earlier.[5] Analysis by starch-gel electrophoresis[6] or chromatography on IRC-50[7] indicated that over 95% of the protein in hemolysates was hemoglobin. For this reason, hemolysates were used without further purification.

Peptide patterns: Hemolysates were digested with trypsin,[9] and the resulting soluble tryptic peptides fingerprinted on paper[10] or analyzed on columns as described by Jones.[11] Tracings of the fingerprints are presented in Figure 1. Several fingerprints were made from each digest and sprayed with ninhydrin reagent or reagents that specifically detect peptides containing histidine, arginine, tryptophan, or tyrosine.[10] It was possible to judge whether peptides in the animal fingerprints corresponded to specific human peptides from the chromatographic and electrophoretic behavior of the animal peptides as well as from the nature of the reaction with the specific spray reagents.

Several chromatograms which have been obtained by the column chromatographic method are shown in Figure 2. In this method, the tryptic peptides which are soluble at pH 3 are resolved chromatographically on 0.9 × 15 cm columns of Dowex-50, ×8, ion exchange resin with a linear gradient formed with 0.2 *M* pyridine acetic acid, pH 3.1, as initial buffer, and 2 *M* pyridine acetic acid, pH 5.0, as limit buffer. The peptides from the column are detected with ninhydrin in the automatic amino acid analyzer system of Spackman, Moore, and Stein.[8] In this method, the peptides are identified on the basis of the volume of buffer required to elute the peptide from the column.

From the results of the two fingerprinting methods it is possible to determine which peptides of the α and β chains of each animal hemoglobin differ from analogous peptides in human hemoglobin. In the majority of cases, the two methods yielded the same information. In certain instances, one of the methods of analysis clearly showed that a peptide differed from the analogous peptide in the human molecule, whereas the second method failed to show a difference. This is seen in the case of the peptides from the globin of *Hylobates lar*. It is impossible to detect a difference between these hemoglobins by fingerprinting on paper (Fig. 1), but on column analysis (Fig. 2) it is evident that the peak corresponding to βTp-IX of the human is not found (95 ml).

TABLE 1

COMPARISON OF α CHAIN PEPTIDES IN VARIOUS PRIMATE HEMOGLOBINS

Species	Tryptic Peptides*										A/A + N
	αTp-I	αTp-I,II	αTp-III	αTp-IV	αTp-V	αTp-VI	αTp-VII	αTp-IX	αTp-X	αTp-XIV	
Hylobates lar	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	0/10
Cacajao rubicundus†	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	0/10
Saimiri sciurea†	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	0/10
Macaca mulatta	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	0/10
Cercopithecus mitis†	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	0/10
Papio doguera	$A_{p,c}$	$A_{p,c}$	$N_{p,c}$	$N_{p,c}$	$A_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	3/10
Galago crassicaudatus	$N_{p,c}$	$N_{p,c}$	A_c	N_p	$N_{p,c}$	$N_{p,c}$	A_c	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	2/10
Perodicticus potto	$N_{p,c}$	$N_{p,c}$	A_c	N_p	$N_{p,c}$	$N_{p,c}$	A_c	$N_{p,c}$	$N_{p,c}$	$N_{p,c}$	2/10
Propithecus verreauxi†	A_p	A_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	2/10
Lemur variegatus†	A_p	A_p	N_p	A_p	N_p	N_p	N_p	N_p	N_p	A_p	4/10
Lemur catta	A_c	A_c	A_c	A_p	$N_{p,c}$	$N_{p,c}$	A_c	N_p	$N_{p,c}$	$A_{p,c}$	6/10
Lemur fulvus	A_c	A_c	A_c	N_p	$N_{p,c}$	$N_{p,c}$	A_c	N_p	$A_{p,c}$	$A_{p,c}$	6/10
Tupaia glis†	A_p	A_p	N_p	A_p	N_p	A_p	N_p	A_p	A_p	A_p	7/10
Total number of amino acids per peptide	7	11	5	15	9	16	4	29	2	2	

* N denotes those peptides indistinguishable from analogous human peptides; A denotes differences in behavior from analogous human peptides. Subscripts p and c refer to behavior in paper and column systems, respectively.
† Hydrolysates from these species have not been analyzed by the column method.

TABLE 2

COMPARISON OF β CHAIN PEPTIDES IN VARIOUS PRIMATE HEMOGLOBINS

Species	Tryptic Peptides* βTp-I	βTp-II	βTp-III	βTp-IV	βTp-V	βTp-VI	βTp-VII	βTp-IX	βTp-XII	βTp-XIII	βTp-XIV	βTp-XV	A/A + N
Hylobates lar	$N_{p,e}$	$N_{p,e}$	$N_{p,e}$	N_p	$N_{p,e}$	$N_{p,e}$	$N_{p,e}$	A_e	N_p	N_p	N_p	$N_{p,e}$	1/12
Cacajao rubicundus†	N_p	N_p	A_p	N_p	A_p	N_p	N_p	N_p	N_p	?	N_p	N_p	2/11
Saimiri sciurea†	N_p	N_p	A_p	N_p	N_p	N_p	N_p	N_p	N_p	?	N_p	N_p	1/11
Macaca mulatta	$N_{p,e}$	$N_{p,e}$	$N_{p,e}$	N_p	$N_{p,e}$	$N_{p,e}$	$N_{p,e}$	A_e	$N_{p,e}$	$N_{p,e}$	$N_{p,e}$	$N_{p,e}$	2/12
Cercopithecus mitis†	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	N_p	0/12
Papio doguera	$A_{p,e}$	$A_{p,e}$	$A_{p,e}$	N_p	A_p	$N_{p,e}$	$A_{p,e}$	A_e	N_p	?	A_p	A_e	8/11
Galago crassicaudatus	$A_{p,e}$	$N_{p,e}$	$A_{p,e}$	N_p	$A_{p,e}$	$N_{p,e}$	$N_{p,e}$	$N_{p,e}$	?	?	N_p	$N_{p,e}$	3/10
Perodicticus potto	$A_{p,e}$	$A_{p,e}$	$A_{p,e}$	N_p	$A_{p,e}$	$N_{p,e}$	$N_{p,e}$	A_e	?	?	N_p	$N_{p,e}$	7/10
Propithecus verreauxi†	A_p	A_p	A_p	N_p	A_p	N_p	A_p	?	?	?	A_p	N_p	6/10
Lemur variegatus†	A_p	A_p	A_p	N_p	A_p	N_p	N_p	?	?	?	A_p	N_p	5/9
Lemur catta	$A_{p,e}$	A_p	$A_{p,e}$	N_p	$A_{p,e}$	$N_{p,e}$	$N_{p,e}$	A_e	?	?	A_p	$N_{p,e}$	6/10
Lemur fulvus	$A_{p,e}$	A_p	$A_{p,e}$	N_p	$A_{p,e}$	$N_{p,e}$	$N_{p,e}$	A_e	?	?	A_p	$N_{p,e}$	6/10
Tupaia glis†	A_p	A_p	A_p	N_p	A_p	N_p	N_p	?	?	?	A_p	A_p	6/9
Total number of amino acids in peptide	8	9	13	10	19	2	4	16	16	12	12	2	

*, † For explanation see legend for Table 1.

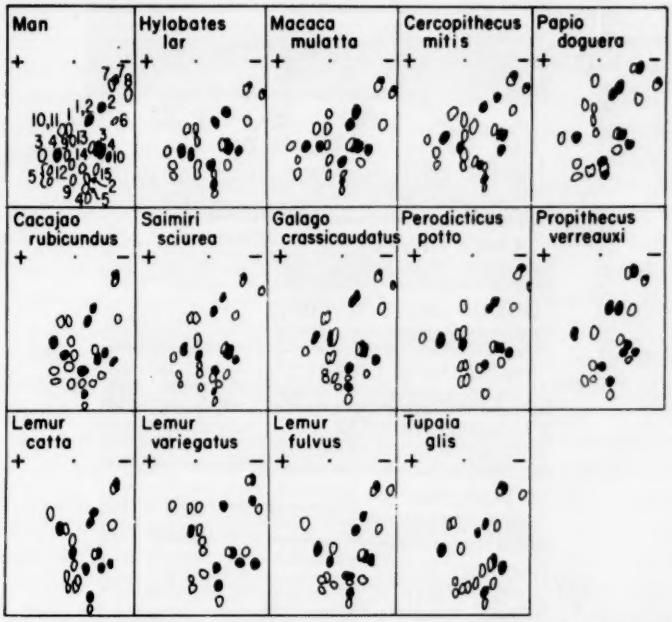

FIG. 1.—Tracings of peptide patterns of several primate hemoglobins. The peptides in human hemoglobin are numbered according to the recently proposed nomenclature.[12] Peptides from α chains are black, and those from β chains are white.

The peak at 130 ml in the gibbon chromatogram is much broader than that of the human and presumably contains the aberrant peptide, βTp-IX. Analysis of the data in Figures 1 and 2 by this means has allowed estimation of the behavior of analogous peptides. The differences observed are summarized in Tables 1 and 2. It should be emphasized that a final comparison of α and β chain sequences can come only from a detailed analysis of the order of amino acids in purified peptides. On the other hand, valuable information can be obtained when 2 methods, such as those used here, are available.

Tryptic peptides from α and β chains: Globins prepared from hemoglobin were separated into α and β chains by countercurrent distribution[13] or by urea-gradient chromatography on IRC-50.[14] The amino acid composition of each chain was determined by chromatographic analysis of acid

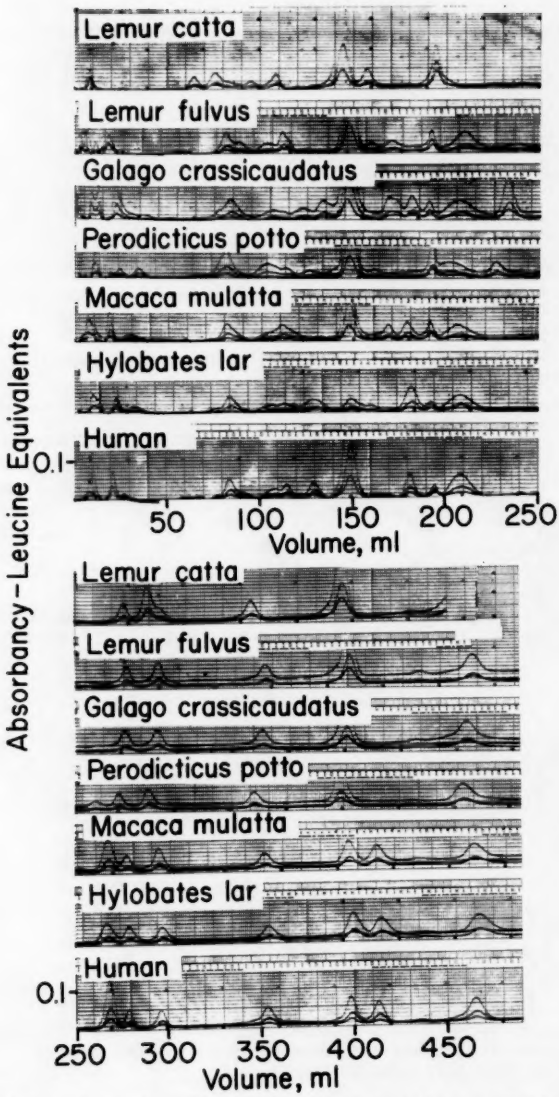

FIG. 2.—Analysis by column chromatography of tryptic hydrolysates of globin from several primates, according to the method of Jones.[11]

hydrolysates according to the methods of Spackman *et al.*[8] and Moore.[15] Although space does not permit tabulation of amino acid compositions, it is noteworthy that the differences in composition of analogous chains between prosimian primates and man are greater than differences in composition between Anthropoidea and man. The most striking feature is the occurrence of 1–3 residues of isoleucine per mole of both α and β chain in lemur hemoglobin.

The tryptic peptides from the α and β chains of several globins were separated chromatographically on Dowex-50.[16] The purity of the peptides was determined by paper chromatography

in two solvent systems. The amino acid compositions of several pure peptides from β chains are given in Table 3. Although the sequences of these peptides must be established, it is convenient to present the compositions in terms of the sequences of analogous human β and γ chain peptides. The following kinds of reasoning led to the sequences presented. There are seldom more than 3–4 amino acids, and often less, in any one peptide which are not present in the analogous human β chain peptide. Thus, to obtain a tentative sequence, those amino acids which were common to both the animal and human peptides were placed at positions which have been established for these amino acids in the human peptide. To place the other residues, advantage was taken of the fact that many of the amino acids which have no counterpart in β chains were present in human γ chains. It was thus possible to write most of the sequences in this manner without the necessity of placing more than one or two residues that were not present in either human γ or β chains. Inspection of the sequence for βTp-I in *Galago crassicaudatus* demonstrates how this was done. The five residues which are found in βTp-I of man were assigned to positions 1, 2, 4, 5, and 8. Phenylalanine and aspartic acid were assigned to positions 3 and 7 so as to correspond to the location of these residues in γTp-I. Glycine, the only remaining residue not defined by γ or β chains, was placed in position 6. Similar arguments were used for the other sequences. Although it is evident that this type of reasoning gives no assurance as to the exact sequence of these peptides, it is remarkable how few residues cannot be placed by analogy with β or γ chains.

Discussion.—The number of structural differences between the hemoglobin of man and the hemoglobin of another primate seems to be proportional to the presently accepted evolutionary relationship between man and the lower primates under consideration. *Hylobates lar*, the great ape studied most extensively by us, possesses a hemoglobin which differs only slightly in structural detail from human hemoglobin. This is in accord with studies on the hemoglobins from the other great apes.[2] The hemoglobins from three Old World monkeys (*Macaca mulatta*, *Cercopithecus mitis* and *Papio doguera*) as well as two South American monkeys (*Saimiri sciurea* and *Cacajao rubicundus*) also are quite similar to human hemoglobin, although they differ to a greater extent than the hemoglobins of the great apes differ from that of man. The hemoglobins from *Galago crassicaudatus* and *Perodicticus potto* (African lorises) differ to an even greater extent, although not so much as the hemoglobins from the more primitive lemurs and the most primitive primate which was examined, an arboreal tree shrew (*Tupaia glis*).

It is evident that there is a greater variation in β chains than α chains throughout the order. The β chains of any one animal differ from human β chains to a greater extent than the α chains of the same animal differ from human α chains. The peptide patterns on paper (Fig. 1) show this most strikingly. The last column in Tables 1 and 2 shows the ratio of the number of peptides which differ from analogous human peptides to the total number of peptides observed. Differences were not found in the α chains of the gibbon, the two South American monkeys, and two of the three Old World monkeys, whereas one or two differences occur in the β chains of each of these species. At least three α chain peptides from *Papio doguera* differ from analogous human peptides. This seems to be an exception to the constancy of α chain structure among man, the higher apes, and the monkeys, but 9 of 11 β chain peptides from *Papio doguera* differ from analogous peptides in man. This reflects the pattern among the α and β chains from the more primitive primates. *Perodicticus potto*, *Galago crassicaudatus*, and the lemurs have peptides in their α chains which differ from analogous human peptides, but in each species there are a larger number of β chain peptides which differ from their human analogs. In the case of *Tupaia glis*, it is striking that only a few α or β chain peptides can be distinguished as analogous to human sequences.

Despite the apparent "instability" of β chains during evolution, it is evident that certain sequences in these chains do not show great variation. This is demonstrated by residues 30–40 having identical compositions in all primate hemoglobins which have been examined at this time. This stability might suggest that this sequence makes an important contribution to the function of the molecule, and mutations which affect this region have not survived.

Consideration of the compositions of the primate peptides shown in Table 3

TABLE 3

THE AMINO ACID COMPOSITION OF SOME PRIMATE TRYPTIC PEPTIDES FROM β CHAINS*

	1 5 (βTp-I) 18 20 25 (βTp-III)
Human β chain†	Val-his-leu-thr-pro-glu-glu-lys . . . . . val-asg-val-asp-glu-val-gly-gly-glu-ala-leu-gly-arg
Hylobates lar	(Val,his,leu,thr,pro,glu,glu,lys) . . . . . (val,asp,val,asp,glu,val,gly,gly,glu,ala,leu,gly,arg)
Papio doguera	. . . . . (val,asp,val,asp,glu,val,gly,gly,glu,ala,leu,gly,arg)
Galago crassicaudatus	(Val,his,*phe*,thr,pro,*gly*,*asp*,lys) . . . . . (val,asp,val,*glu*,glu,val,gly,gly glu,ala,leu,gly,arg)
Perodicticus potto	(Val,his,leu,thr,*glu*,*gly*,*asp*,lys) . . . . . (val,asp,val,asp,glu,val,gly,gly,glu,ala,leu,gly,arg)
Propithecus verreauxi	. . . . . (val,asp,val,*glu*,*asp*,*ala*,gly,gly,glu,*thr*,leu,gly,arg)
Lemur variegatus	. . . . . (val,asp,val,*glu*,*lys*)
L. catta	. . . . . (val,asp,val,*glu*,*lys*,val,gly,gly,glu,*thr*,leu,gly,arg)
L. fulvus	. . . . . (val,asp,val,*glu*,*lys*,val,gly,gly,glu,ala,leu,gly,arg)
	(γTp-I) (γTp-III)
Human γ chain‡	Val-his-*phe*-thr-*glu*-glu-*asp*-lys . . . . . val-asg-val-*glu*-*asp*-*ala*-gly-gly-glu-*thr*-leu-gly-arg-
	31 35 (βTpIV) 45 50
Human β chain	leu-leu-val-val-tyr-pro-try-thr-glu-arg-phe-phe-glu-ser-phe-gly-asp-leu-ser-thr-pro-
Hylobates lar	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)(phe,phe,glu,ser,phe,gly,asp,leu,ser,thr,pro,
Papio doguera	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)(phe,phe,glu,ser,*leu*,gly,asp,leu,*glu*,thr,pro,
Galago crassicaudatus	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)(phe,phe,glu,ser,*leu*,gly,asp,leu,ser,thr,pro,
Perodicticus potto	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)
Propithecus verreauxi	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)(phe,phe,glu,ser,phe,gly,asp,leu,ser,*ser*,pro,
Lemur variegatus	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)(phe,phe,glu,ser,phe,gly,asp,leu,ser,*ser*,pro,
L. catta	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)
L. fulvus	(leu,leu,val,val,tyr,pro,try,thr,glu,arg)
	(γTp-IV)
Human γ chain	leu-leu-val-val-tyr-pro-try-thr-glu-arg-phe-phe-*asp*-ser-phe-gly-*asg*-leu-ser-*ser*-*ala*-
	55 (βTp-V) (βTp-VI) (βTp-VII) 70
Human β chain	asp-ala-val-met-gly-asg-pro-lys-val-lys-ala-his-gly-lys-lys-val-leu-gly-ala-phe-ser-
Hylobates lar	asp,ala,val,met,gly,asp,pro,lys)(val,lys)(ala,his,gly,lys)
Papio doguera	asp,ala,val,met,gly,asp,pro,lys) (val,leu,gly,ala,phe,ser,
Galago crassicaudatus	*glu*,ala,val,met,gly,asp,pro,lys)(val,lys)(ala,his,gly,lys) (val,leu,*glu*,*ser*,phe,ser,
Perodicticus potto	(val,lys) (val,leu,*thr*,*ser*,phe,*gly*,
Propithecus verreauxi	*ser*,ala,*ileu*,met,gly,asp,pro,lys)(val,lys) (val,leu,gly,*ser*,phe,ser,
Lemur variegatus	*ser*,ala,*ileu*,met,gly,asp,pro,lys)(val,lys) (val,leu,*thr*,*ser*,phe,*gly*,
L. catta	(val,lys)(ala,his,gly,lys)
L. fulvus	(val,lys)
	(γTp-V) (γTp-VI) (γTp-VII)
Human γ chain	*ser*-ala-*ileu*-met-gly-asp-pro-lys-val-lys-ala-his-gly-lys- val-leu-*thr*-*ser*-*leu*-*gly*-
	75 80 (βTp-IX)
Human β chain	asp-gly-leu-ala-his-leu-asp-asp-leu-lys
Hylobates lar	
Papio doguera	asp,*glu*,leu,ala,his,leu,asp,asp,leu,lys)
Galago crassicaudatus	asp,*asp*,leu,ala,his,leu,asp,asp,leu,lys)
Perodicticus potto	asp,*ala*,*val*,ala,his,leu,asp,asp,leu,lys)
Propithecus verreauxi	asp,*ala*,leu,*glu*,his,leu,asp,asp,leu,lys)
Lemur variegatus	glu,*glu*,*thr*,*pro*,his,leu,asp,asp,leu,lys)
L. catta	
L. fulvus	
	(γTp-IX) (γTp-X)
Human γ chain	asp-*ala*-*ileu*-*lys*-his-leu-asp-asp-leu-lys

* The residues in italics are those which differ from analogous residues in human β chains.
† Sequence according to Braunitzer *et al.*[18]
‡ Sequence according to Schroeder *et al.*[19]

reveals a striking similarity between the β chains of primitive primates and human γ chains. The human peptides βTp-I (or γTp-I) through βTp-IX (or γTp-X) account for 83 residues of the human γ and β chains. In these portions of the two human chains, there are 20 differences in amino acid sequence (residues italicized). In only 4 of the 39 peptides from various primates was it necessary to assign an amino acid to a position other than one of the 20 positions where β and γ chains differ in sequence (glycine at position 6 in βTp-I of *Perodicticus potto* and *Galago crassicaudatus;* leucine at position 45 in βTp-V of *Papio doguera* and *Galago crassicaudatus*). Thus, most of the differences in composition of the primate peptides can be placed

in one of the 20 positions where β and γ chains differ. Furthermore, when the composition of one of the peptides differed from β chains it was evident that the amino acids which differed were residues that were unique to γ chains. Table 4 shows the number of amino acids in the 20 unique positions which are "β-like," the

TABLE 4

DISTRIBUTION OF "β-LIKE" AND "γ-LIKE" REPLACEMENTS IN SOME PRIMATE β CHAINS

Species	β-like*	γ-like*	Neither γ- nor β-like*
Hylobates lar	13/13	0/13	0/13
Papio doguera	15/18	0/18	3/18
Galago crassicaudatus	12/20	3/20	5/20
Perodicticus potto	8/15	6/15	1/15
Propithecus verreauxi	5/15	9/15	1/15
Lemur variegatus	2/12	7/12	3/12

* The ratios are the number of replacements of the kind indicated to the total number of replacements observed in the peptides examined for the animal indicated (see Table 3).

same as β chains; "γ-like," the same as γ chains; or neither of these types. In agreement with the results from peptide patterns (Fig. 1), *Hylobates lar* contains only those amino acids which are unique to β chain in these 20 positions. *Papio doguera* has 15 identical to β chain and 3 which do not correspond to β or γ chains. In the African lorises and the lemurs, fewer of the 20 positions can be filled by amino acids that are unique to β chains whereas an increasing number are filled by the amino acids found in γ chains. This is particularly noteworthy for isoleucine which is absent in β chains but occurs at position 54 in γ chains and presumably at the same position in β chains of *Propithecus verreauxi* and *Lemur variegatus*.

Although sequence analysis of the peptides from these lemurs will provide a final proof of the "γ-like" structure, it is interesting to note that of all of the primate hemoglobins examined at this time, only human fetal hemoglobin and the hemoglobin from certain shrews, lemurs, and other prosimians are resistant to alkaline denaturation.[17] The exact structural properties which render a hemoglobin resistant (or sensitive) to alkali are unclear, but resistance must reside in features of the primary structure of the γ chain.

From these observations we have arrived at the following conclusions concerning the evolutionary relationships among the polypeptide chains of primate hemoglobins: (1) α chains are more stable than β chains during the evolution of primates; and (2) the β chains of lower primates contain many amino acids which are present in γ chains of man.

These conclusions are in surprisingly good agreement with the scheme of evolution for the hemoglobin chains which has been proposed by Ingram,[1] and Zuckerkandl and Pauling.[3] In these schemes it is suggested that the four kinds of chains in human hemoglobin evolved from a single primitive myoglobin-like heme protein by repeated gene duplication and translocation. Although these papers should be consulted for details, a general scheme taken from these authors is shown in Figure 3.

The apparent stability of α chains to sequence variation throughout evolution was predicted on the basis that this chain is required to fit structurally two different partners, β chains and γ chains. Thus, alteration of α chains would influence not only the nature of the adult hemoglobin molecule but also the critical hemoglobin of fetal life.

The evolution of β and γ chains from a primitive ancestor was also suggested.

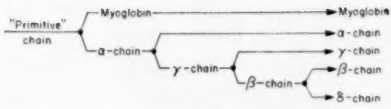

FIG. 3.—Scheme for the evolution of hemoglobin chains, after Ingram.[1]

Zuckerkandl and Pauling[3] have estimated that the common ancestor of the α and γ chains occurred some 600×10^6 years ago, the γ and β ancestor 260×10^6 years ago, and the β and δ ancestor about 44×10^6 years ago. Inasmuch as mammalian evolution, including that of primates, has been going on for some 160×10^6 years, one would expect analysis of primate hemoglobins to shed light only on the most recent stages of the evolution of hemoglobins. It is reasonable to suggest from the data presented here that "γ-like" chains occur in primitive primates. If this is the case, then the estimated time of derivation of γ and β chains from a common ancestor (260 million years) is possibly an overestimate, and β chain derivation from a "γ-like" precursor might have taken place within the 60 million years of mammalian evolution. On the other hand, evaluation of sequences in the polypeptide chains of the lower mammal groups might allow a more exact determination of the origin of a common β-γ chain ancestor. Since the chains of adult prosimians have a γ-like character, it would also be helpful to determine the sequences of the γ chains in the fetal hemoglobin from prosimians. It can be anticipated that the structures of such chains might shed light on the β-γ-common ancestor chains. As human γ chains reflect adult, primitive primate chains, so might the fetal prosimian chains reflect an even more primitive type of chain.

There seems to be little question that the δ chains have their origin within the period of primate evolution. It is surprising, however, that the data presented here give little indication of the origin of these chains. Certainly some primates lack components analogous to HbA_2 while the great apes (and others) seem to possess such hemoglobins.[6] It will be helpful to compare the sequences in these chains with analogous sequences in the adult components, and in this way some data bearing on the origin of a β-δ ancestor chain might be obtained. The compositions of the primate peptides given in Table 3 cannot be interpreted to show "δ-like" replacements.

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* The system of nomenclature recently proposed for the tryptic peptides from human hemoglobins will be used in this paper.[12]

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*CHROMOSOMALLY DIRECTED PROTEIN SYNTHESIS**

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The different kinds of specialized cells of a higher organism differ among themselves in the kinds of enzymes and other proteins which they contain. Nonetheless, each possesses in the chromosomes of its nucleus the complete genomal DNA containing information concerning the manufacture of all of the kinds of enzymes contained in all of the kinds of cells of that creature. It is clear, therefore, that there is in the nucleus some mechanism for the control of genetic activity, a mechanism responsible for that orderly repression and derepression of gene activity which makes possible development. The present work concerns the control of genetic activity.

Genetic activity consists in the direction of DNA-dependent RNA synthesis, the RNA thus formed containing in coded form the message appropriate for the ribosomal synthesis of a particular protein. For the study of the control of genetic activity, we first establish an *in vitro* system for the conduct of chromosome-dependent RNA synthesis. We then couple our chromosomal RNA-generating system to a messenger RNA-dependent ribosomal protein synthesis system.

With our coupled system established we next focus attention upon a particular gene and its product-protein, in our case the gene of the pea plant responsible for the production of the reserve globulin of pea cotyledons. This protein is synthesized in developing pea cotyledons, and is not synthesized by such other pea plant tissues as buds or roots. It will be shown that a specific protein, pea seed globulin, is synthesized by our coupled system in response to the presence of cotyledon chromatin, and is not synthesized in response to the presence of pea bud chromatin. Thus, the control of genetic activity characteristic of the living cell is preserved in the isolated chromatin. It will be further shown that such control is exerted by the histone component of the chromosome.

Materials and Methods.—Preparation of chromatin: Chromatin was prepared by the methods of Huang and Bonner.[1] The organ or tissue (in this work, of pea plants, var. Alsaka) is first

ground in a blendor, in a grinding medium composed of sucrose, 0.25 *M*; tris, pH 8, 0.05 *M*; $MgCl_2$, 0.001 *M*. The homogenate after filtration through cheesecloth and miracloth is centrifuged at 4,000 × *g* for 30 min and the supernatant discarded. The crude chromatin, which contains 90–95% of the tissue DNA, is scraped from the underlying starch pellet, resuspended in 0.05 *M* tris, pH 8, and repelleted for 15 min at 10,000 × *g*. This step is repeated twice. The crude chromatin is then layered on 1.9 *M* sucrose and centrifuged at 22,000 rpm for 120 min in the SW-25 Spinco head. The pellet, resuspended in dilute saline citrate (0.016 *M*), constitutes the purified chromatin which has been freed of contaminating protein by this procedure. In the case of chromatin of pea buds, we have found it advantageous to use the crude chromatin dialyzed against dilute saline citrate without further purification.

RNA polymerase: RNA polymerase was prepared from log phase cells of *E. coli* strain B by the method of Chamberlin and Berg[2] and purification of the enzyme carried through ammonium sulfate fractionation to their fraction 3. The freshly made enzyme at this stage possessed a specific activity of approximately 160 mμm AMP incorporated into RNA/10 min/mg enzyme protein, a dependency ratio (activity in presence of added DNA/activity in absence of added DNA) of 40- to 100-fold and a 280/260 ratio of 1.2 to 1.5.

Preparation of ribosomes: The *E. coli* ribosomal system was prepared from cells ground with alumina according to the procedure of Nirenberg and Matthaei.[3] The resulting homogenate was centrifuged at 30,000 × *g* for 60 min and the supernatant recentrifuged for a further 20 min. This supernatant was then incubated for 45 min at 37°C in the presence of a complete protein synthesis reaction mixture, including 19 amino acids, ATP, and phosphoenolpyruvate and its kinase. Ribosomes were then pelleted from the preincubation mixture, resuspended, repelleted, and finally resuspended and exhaustively dialyzed. The supernatant from the initial ribosomal pellet, containing activating and other soluble enzymes, as well as *E. coli* transfer RNA, was exhaustively dialyzed and then freed of *E. coli* DNA and its RNA polymerase by 18 hr of centrifugation at 105,000 × *g*. We have found the optimum composition of the ribosomal system to consist of, per 50 μg ribosomal protein, 70 μg protein of supernatant fraction supported by 20 mμm of RNA generated by the *E. coli* polymerase system.

Analytical procedures: Analysis of RNA, DNA, and protein were done by the methods standard to our laboratory.[1, 4] Determination of radioactivity incorporated into RNA or protein was accomplished by 5× repeated washing of the sample with cold 10% TCA, after which it was dissolved in 0.2 *N* NH_4OH, and pipetted on to and dried on a glass planchet for counting.

Immunochemical detection of pea seed globulin: Authentic pea seed globulin was prepared from mature pea seeds, var. Alaska, by the method of Danielsson.[5] Potent antisera were developed in chickens by repeated immunization with the globulin over a period of six months. A single lot of pooled antiserum was used for all of the work here reported. The amount of antiserum required to maximally precipitate a given amount of globulin (slight antibody excess) was determined by standard quantitative precipitin methods and used thereafter. Synthesis of globulin both *in vivo* and by the *in vitro* ribosomal system was detected as follows: to the reaction mixture containing soluble C^{14}-labeled proteins, 0.5 mg of authentic carrier globulin (except in the case of pea cotyledons *in vivo*, which contain endogenous globulin) was added the appropriate amount of antiserum protein. Precipitate formation was then allowed to develop maximally (1 hr at 37°C). The precipitated complex, containing approximately 2.5 mg of protein (ratio of pea globulin to antibody, 1:3), was centrifuged off and washed by five successive complete resuspensions and recentrifugations in cold 0.15 *M* NaCl. The complex was then dissolved in 0.2 *N* NH_4OH and counted.

Experimental Results.—Isolated chromatin possesses the ability to conduct DNA-dependent RNA synthesis, using as substrate the four riboside triphosphates,[6] and contains bound chromosomal RNA polymerase.[1] However, chromatin, pretreated at 60°C for 5 min to inactivate its endogenous polymerase and other enzymes so that it can serve only as template, can support RNA synthesis by the purified polymerase of *E. coli*, as is shown in Table 1. In addition, the RNA synthesis supported by chromatin in response to added *E. coli* RNA polymerase consists of molecules which are largely free, that is, do not remain bound to and sedi-

TABLE 1

ACTIVITY OF CHROMATIN OF DEVELOPING PEA COTYLEDONS AND OF PEA DNA IN SUPPORT OF RNA SYNTHESIS BY *E. coli* RNA POLYMERASE

System*	RNA synthesis ($\mu\mu$m nucleotide/10 min)
125 μg deproteinized DNA: *coli* polymerase present	2,220†
37.5 μg deproteinized DNA: *coli* polymerase present	2,030
12.5 μg deproteinized DNA: *coli* polymerase present	740
125 μg DNA in form of native chromosomal nucleohistone: *coli* polymerase present	25
125 μg DNA as whole chromatin: *coli* polymerase present	760
125 μg DNA as whole chromatin heated at 60°C for 5 min: *coli* polymerase present	940
125 μg DNA as whole chromatin: no added *coli* polymerase	17

* Reaction mixture includes tris, pH 8, 20 μm; $MnCl_2$, 0.5 μm; $MgCl_2$, 2 μm; 8-C^{14}-ATP, 0.13 μm, 1.5 μc/μm; GTP, CTP, and UTP each 0.2 μm; β-mercaptoethanol, 6 μm; and chromatin or DNA and *E. coli* polymerase (approx. 10 μg) as indicated, all in 0.5 ml final volume. Incubation at 37°C.
† Incorporation by polymerase alone subtracted.

ment with the chromatin, as does RNA synthesized by chromosomal RNA polymerase.[7]

The data of Table 1 show not only that whole chromatin, in this case of developing pea cotyledons, supports RNA synthesis by *E. coli* RNA polymerase, but that, in addition, the nucleohistone component of such chromatin prepared by the methods of Bonner and Huang[4] and in which DNA and histone are complexed in very nearly equivalent amounts is almost totally inactive in the support of RNA synthesis. Since this nucleohistone component (in which the histone greatly stabilizes the DNA against melting[4]) includes the bulk of the DNA of pea cotyledon chromatin, only a small fraction of the DNA of such chromatin would appear to be present in an active state. The data of Table 1 show in fact that under conditions in which DNA is limiting, the DNA of pea cotyledon chromatin is as active in the support of RNA synthesis as about one-tenth as much deproteinized whole genomal pea DNA.

Chromatin-dependent RNA synthesis will next be coupled to a messenger RNA-dependent ribosomal system. For this purpose ribosomes are borrowed from *E. coli*.[8] The mode of their preparation, described under *Materials and Methods*, makes the system highly dependent upon exogenous messenger RNA. Toward the achievement of this end, preincubation of the crude system in a complete protein synthesis reaction mixture contributes importantly, as is shown in Table 2. Protein synthesis by the ribosomal system is highly dependent, as shown in Table 3, upon both added DNA and upon added RNA polymerase. Pea plant DNA is as effective in this function as is that of bacteriophage T2 or T4. The protein synthesized by the ribosomal system in response to RNA formed by the DNA-depend-

TABLE 2

DEPENDENCY OF RIBOSOMAL PROTEIN SYNTHESIS SYSTEM ON EXOGENOUS MESSENGER RNA AS AFFECTED BY PREINCUBATION OF RIBOSOMAL SYSTEM IN COMPLETE PROTEIN SYNTHESIS REACTION MIXTURE

System*	C^{14}-Leucine Incorporation into Protein ($\mu\mu$m/60 min/mg ribosomal protein)	
	Preincubated	Not preincubated
Ribosomal system alone	88	251
Ribosomal system + pea DNA + RNA polymerase	844	754

* Reaction mixture contains tris, pH 8, 50 μm; $MgAc_2$, 2 μm; $MnCl_2$, 0.5 μm; KCl, 20 μm; β-mercaptoethanol, 3 μm; 18 amino acids, each 0.02 μm; C^{14}-leucine, 6.8 μc/μm, 0.075 μm; ATP(K), 1 μm; GTP, CTP, UTP each 0.1 μm; 50 μg ribosomal protein; 69 μg 105,000 $\times$ *g* supernatant protein; pea DNA, 100 μg; and approx. 20 μg *E. coli* RNA polymerase, all in total volume of 0.3 ml. Incubation at 37°C.

TABLE 3

DEPENDENCE OF PROTEIN SYNTHESIS BY *E. coli* RIBOSOMAL SYSTEM ON PEA AND *E. coli* RN POLYMERASE

Additions to ribosomal system*	C^{14}-Leucine Incorporation into Protein ($\mu\mu$m/mg ribosomal protein/30 min)
Pea DNA, 125 μg; RNA polymerase	1,158
T2 DNA, 125 μg; polymerase	828
T4 DNA, 125 μg; polymerase	756
Polymerase; no DNA	100
Pea DNA, 125 μg; no polymerase	102
No DNA; no polymerase	104

* Reaction mixture includes components specified in Table 2.

TABLE 4

SUPPORT OF RIBOSOMAL PROTEIN SYNTHESIS BY CHROMATIN-DEPENDENT RNA SYNTHESIS*

System†	C^{14}-Leucine Incorporation into Protein (cpm/30 min)	
	Two-step expt.	One-step expt.
Cotyledon chromatin; RNA polymerase: ribosomal system	505	721
Cotyledon chromatin; ribosomal system: no polymerase	87	133
Bud chromatin + RNA polymerase: ribosomal system	182	924
Bud chromatin; ribosomal system: no polymerase	122	110

* In the two-step experiment, RNA is generated by chromatin for 30 min, the chromatin removed by centrifugation, and the ribosomal system added to supernatant. In the one-step experiment, all ingredients are present simultaneously.

† Reaction mixture includes components specified in Table 2 except for C^{14}-leucine. In this case, uniformly labeled leucine, 0.02 μm, 131 μc/μm, was used. Chromatin containing 250 μg DNA used in each case.

ent RNA generating system is largely soluble, i.e., is released from the ribosomes. Of the protein synthesized, 65–90 per cent (av. 75 per cent) remains in solution after removal of the ribosomes by centrifugation.

We now couple chromatin-dependent RNA synthesis to messenger RNA-dependent protein synthesis. This may be accomplished in two steps, as is shown by the data of Table 4. In the first step chromatin and RNA polymerase synthesize RNA. The chromatin is then centrifuged off at 16,000 × *g* and the ribosomal system added to the RNA-containing supernatant. As the data of Table 4 also show, however, it is more effective to simply combine chromatin, polymerase, and ribosomal system, and thus to directly couple RNA synthesis to protein synthesis. This is particularly true for the case of protein synthesis supported by bud chromatin.

We next focus upon the particular gene (in fact, genes[5]) and its product-protein, pea seed globulin, which we have selected for study. We consider first the extent to which globulin is synthesized by varied tissues of the plant. For the experiment of Table 5, 1- to 10-gm samples of each of a variety of pea plant tissues were ex-

TABLE 5

SYNTHESIS OF PEA SEED GLOBULIN *in vivo* BY VARIED ORGANS OF THE PEA PLANT

Organ	C^{14}-Leucine Incorporation into Protein		
	Total soluble protein (cpm)	Globulin-antibody precipitate (cpm)	Globulin/total protein (%)
Flower	17,500	510	2.9
Cotyledon	7,100	337	4.7
Older cotyledon	9,200	862	9.3
Roots	17,150	31	0.18
Apical bud	11,600	14	0.12
Apical bud	27,600	42	0.15

Tissues were separately incubated in L-leucine (1 μc/ml, 19 μc/μm) for 2 hr at 25°C, in the presence of penicillin, 6 μg/ml. Soluble protein obtained by dialysis of 105,000 × *g* supernatant of tissue homogenate.

cised, and each incubated aerobically with a small volume of buffer containing C^{14}-leucine and penicillin. After 2 hr of incubation, the tissue samples were ground (Virtis homogenizer), filtered through miracloth, and centrifuged for 120 min in the no. 40 Spinco head at 40,000 rpm. The clear supernatants were then freed of C^{14}-leucine by dialysis (four successive changes of 0.1*M* tris buffer, pH 8, 48 hr). To each supernatant representing the soluble proteins of that tissue, authentic carrier globulin and the appropriate amount of high-titer chicken antiserum was added. The specifically precipitated globulin was then centrifuged off, washed as described under *Materials and Methods*, and counted, as were aliquots of the whole supernatant. The data of Table 5 concern then the proportion which globulin-antiserum reacting material, known hereafter as globulin, constitutes of the total protein synthesized by the particular tissue during the experimental period. As is to be expected, globulin constitutes an important protein product of developing pea cotyledons, making up 4–10 per cent of total protein synthesized, the exact amount depending upon the exact stage at which the developing cotyledons are harvested.[5] The flower, too, produces a significant amount of globulin. For such organs as apical buds and root, however, the radioactivity of the globulin-antiserum precipitate constitutes only 0.1–0.2 per cent of total radioactive protein synthesized. A portion, or all, of even this amount may be due to nonspecific contamination of the carrier-precipitate by soluble, labeled but nonglobulin proteins. It may be concluded that pea apical buds synthesize little or no globulin.

For the study of globulin synthesis by the *E. coli* ribosomal system in response to chromosomally generated RNA, incubation mixtures were made on a sufficiently large scale to incorporate 5,000–50,000 cpm of C^{14}-leucine into protein. At the end of the incubation period, the cooled reaction mixtures were centrifuged 120 min in the no. 40 Spinco head at 40,000 rpm for removal of chromatin and ribosomes. The soluble proteins of the supernatant were dialyzed as outlined above. From each such reaction mixture of dialyzed soluble proteins added, authentic globulin was precipitated by the appropriate amount of antibody, just as with the *in vitro* reaction mixtures. The data of the example of Table 6 show that globulin is synthesized by the ribosomal system in response to the presence of chromatin of developing cotyledons. The chromatin of pea buds, although it supports protein

TABLE 6

SYNTHESIS OF PEA SEED GLOBULIN BY RIBOSOMAL SYSTEM OF *E. coli* IN RESPONSE TO VARIED TEMPLATES FOR RNA SYNTHESIS

Template for RNA synthesis	C^{14}-Leucine Incorporation into Protein[a]		
	Total soluble protein (cpm)	Globulin/antibody precipitate (cpm)	Globulin/total protein (%)
Apical bud chromatin[b]	15,650	16	0.10
Apical bud chromatin[c]	41,200	54	0.13
Cotyledon chromatin[c]	23,600	341	1.45
Cotyledon chromatin[c]	14,000	226	1.61
DNA of bud chromatin[d]	15,200	60	0.40
DNA of bud chromatin[d]	14,200	72	0.51
DNA of cotyledon chromatin[d]	5,600	22	0.39
DNA of cotyledon chromatin[d]	50,100	157	0.31

[a] Reaction mixtures included components of Table 2 but on scale 15–40× greater.
[b] Chromatin not subjected to 60°C pretreatment.
[c] Chromatin pretreated at 60°C, 5 min, in dilute saline citrate, 0.015 *M*.
[d] DNA prepared by deproteinization of purified chromatin.

synthesis equally as well as does that of pea cotyledons, does not cause the formation of any globulin detectable above the level of the background.

Although the chromatin of pea buds does not support the synthesis of globulin, the gene or genes responsible for such synthesis is of course present in it. Table 6 includes data from experiments in which protein synthesis by the ribosomal system was supported by the presence of DNA prepared by deproteinization of pea bud or of pea cotyledon chromatin. The amount of radioactivity in the globulin complex prepared from such reaction mixtures, approximately 0.4 per cent of total protein formed, is appreciably greater than that formed either by the pea-bud-chromatin-primed ribosomal system or by pea buds *in vivo*.

Discussion.—We acknowledge that the immunochemical methods here used for the separation and determination of pea seed globulin are not rigorous in the sense that we have not chemically established that the product protein is, in fact, globulin. This is a task for the future. In the present work we are, however, concerned with comparison between the amounts of anti-globulin-reacting material formed in the living cell and in the chromosomally supported ribosomal system, and in the similar comparison between the products of ribosomal systems supported by different chromatins. Used in this manner the immunochemical assay of globulin would be a rigorous one were it not for the background: the small amount of activity associated with the complex when carrier globulin is precipitated with its antibody from protein mixtures containing little or no globulin, as in the case of the proteins of pea buds, pea roots, etc. Whether this background represents nonspecific absorption or a small amount of synthesis of globulin or other cross-reacting material, we do not know. The differences in proportion of globulin formed as between systems supported by cotyledon chromatin, pea bud chromatin, and deproteinized DNA from pea bud or cotyledon chromatin are in any case well beyond the range of this background.

The protein produced by the ribosomal system in response to pea whole-genomal DNA includes a substantial proportion of globulin, in the examples of Table 6, 4 or 5 times above the background. It may be that the genes for synthesis of globulin constitute a larger-than-average proportion of the pea genome. Our immunochemical methods of globulin detection are, however, inadequate to establish this point rigorously.

The results presented above show not only that messenger RNA-dependent protein synthesis can be supported by RNA formed by chromatin-dependent RNA synthesis, but also that during the isolation of chromatin there is preserved to a considerable degree the control of genetic activity characteristic of the particular tissue in life. Cells in which globulin synthesis is repressed yield chromatin which does not support globulin synthesis in our coupled system, and vice versa. It is true, however, that protein synthesis as supported by chromatin of developing pea cotyledons *in vitro* does not result in a proportion of globulin as high as is characteristic of pea cotyledons *in vivo*. This may be due to some derepression of normally repressed genes during the preparation of pea cotyledon chromatin. It may result also from some fractionation of the genome during chromatin preparation, although as is shown in Table 6, the DNA of cotyledon chromatin is approximately as effective as that of pea buds in support of globulin synthesis. It is clear, however, that the repressed state of the genes responsible for globulin synthesis is preserved

during the preparation of pea bud chromatin. What agent is responsible for such repression? It has been shown that removal of protein from pea bud chromatin derepresses the genes for globulin synthesis. The principal protein of chromatin is histone, although other proteins are also present. That it is removal of histone which is responsible for derepression is indicated by the facts (Table 1; see also refs. 1, 4, and 9) that the nucleohistone component of chromatin, in which DNA is fully complexed with histone, is inert in support of RNA synthesis and that ability of chromatin to support RNA synthesis is greatly increased by the specific removal of histone.[1] The histone component of chromatin is, then, a repressor of genetic activity.

Even though we now see that the histone of the chromosome is one agent responsible for genetic repression, the principal questions concerning control of genetic activity remain unanswered. How does nature turn genes off and on—put on or take off histones? How is programming of genetic activity exerted? We cannot yet answer these questions. We do, however, have a system with which such matters can be studied. It appears probable, too, that the present type of system in which the well-standardized, readily preparable components of *E. coli*, such as ribosomes and polymerase, are used as tools for the investigation of chromosomal activity may be of general utility in the study of development in higher organisms.

Summary.—The ability of chromatin, isolated from varied tissues of the pea plant, to support DNA-dependent RNA synthesis is enhanced in the presence of the RNA polymerase of *E. coli*. We have coupled such chromatin-dependent RNA synthesis to a messenger RNA-dependent ribosomal protein synthesis system, the latter also derived from *E. coli*. The material synthesized by the ribosomal system under the direction of chromatin isolated from developing pea cotyledons includes a protein characteristic of such cotyledons, the pea seed reserve globulin. Chromatin of pea buds, which do not synthesize pea seed globulin *in vivo*, does not support the synthesis of such globulin by the isolated ribosomal system. Hence the control of genetic activity characteristic of the living cell is, to an appreciable extent, preserved in the isolated chromatin. This control is exerted by the histone of the chromosome. Thus, the removal of histone from pea bud chromatin, in which the genes for globulin synthesis are repressed, yields DNA which supports globulin synthesis.

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*SOME OBSERVATIONS ON THE ENZYME HYDROGENASE OF DESULFOVIBRIO DESULFURICANS**

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The purification and the properties of hydrogenase of *Desulfovibrio desulfuricans* have been described in earlier papers.[1-4] It was previously observed that both the crude extract and the purified enzyme were completely inactive in the absence of cysteine[1, 2] or dithionite[3] even when these preparations were incubated with hydrogen for several hours. The present communication describes the preparation of hydrogenase which is active without any special activating procedure or additions as assayed by the dye (methylene blue or benzyl viologen) reduction[1, 2] or the deuterium exchange method.[5] This finding is different from the results reported earlier.[1-4] With this preparation it is possible to study the reversibility of the CO inhibition of hydrogenase of *D. desulfuricans* by light and the sulfhydryl nature of the enzyme. Such studies have so far been complicated because of the requirement of dithionite[3] or cysteine[1, 2] for activation. Dithionite prevents reversal of CO inhibition by light.[6]

Experimental.—A phosphate extract of the acetone-treated *D. desulfuricans* (strain Hildenborough, NCIB No. 8303, which is being maintained in lyophilized state in this laboratory) cells was prepared according to the procedure of Sadana and Morey.[2] The crude extract was dialyzed against 0.05 *M* phosphate, pH 7.2, for 48 hr and used without any further purification unless otherwise indicated. Oxygen-free hydrogen was prepared by passage through a Deoxo purifier (Baker and Co., Inc., Newark, New Jersey) and then over hot copper. Carbon monoxide-hydrogen mixtures (v/v) were prepared by mixing the appropriate quantities of H_2 and CO.

The hydrogenase was assayed by the deuterium exchange method[5] or by the methylene blue reduction method[2] at 35°C. The reaction flasks were of either 30-ml or 90-ml volume, depending on the number of gas samples to be removed from the flask. Quartz flask was used for study of the reversibility of CO inhibition by light. The D_2O concentration of the liquid phase was 20%. The pH of the system was 8.0 unless otherwise indicated. The flask was evacuated for 10 min while the solution was frozen in dry ice and then filled with oxygen-free hydrogen or with the required CO-H_2 mixture.

For the experiments on light reversibility, a 2,000-watt bulb (Westinghouse pro-

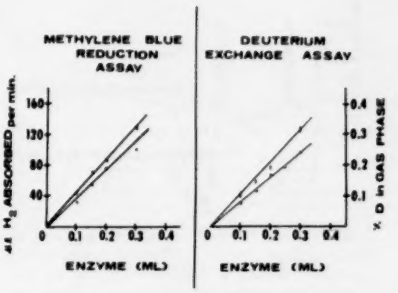

FIG. 1.—Proportionality of hydrogenase activity to enzyme concentration as determined by the methylene blue reduction or by the deuterium exchange method. The activity was measured in the absence and in the presence of cysteine. Deuterium exchange expts: D_2O in liquid phase, 20%; pH 8.0; temp. 35°C. MB reduction expts: pH 8.0; temp., 35°C. Lower line, no cysteine present during measurement of activity; upper line, cysteine present during measurement of activity.

jection bulb PH2M-T48-3AS/EDM) was used. The light was focused onto the reaction vessel with two Fresnel lenses, $14^3/_4$ in. diameter, 14 in. focal length. Between the light source and the first lens there was a large flat bottle filled with cold water to absorb the infrared.

Results.—The data in Figure 1 indicate that in the absence as well as in the presence of cysteine, the enzyme activity is directly proportional to the amount of the enzyme present. In the experiment recorded, the activity without cysteine was approximately 80 per cent of that obtained when cysteine was added. In a number of other preparations, the activity in the absence of cysteine or dithionite varied from approximately 40 to 70 per cent of that observed when cysteine or dithionite was present during the assay. The possibility that the activity observed in the absence of cysteine or dithionite might be due to the presence in the preparation of activating substances seems to be ruled out by the fact that the enzyme retained more than 85 to 90 per cent of its activity on extensive dialysis against phosphate or tris buffers in equilibrium with air. Purification by precipitation of the enzyme at pH 4[2] yields a fraction which is stimulated by the addition of dithionite or cysteine to no greater extent than that observed with the crude dialyzed extract.

The enzyme precipitated from the crude extract at pH 4 could be kept at 0–4°C for at least 24 hr with no detectable loss in activity or enhanced requirement for activating agent. The preparations retained their activity even when kept in contact with air. However, when the enzyme was repeatedly frozen and thawed, it showed more pronounced requirement for activating agents for activity.

pH optimum: It is known from the work of Purec, Krasna, and Rittenberg[6] with the hydrogenase of *Proteus vulgaris* that the enzyme activated by dithionite is somewhat different from the enzyme which is activated spontaneously. With the enzyme from *Desulfovibrio desulfuricans* we observe the pH optimum of the enzyme which is activated either spontaneously or by the addition of cysteine or 2,3-dimercaptopropanol to be at 8, while the dithionite-activated enzyme is most active at 5.6. The pH-activity curves are given in Figure 2. Nearly the same maximal reaction rates were found with the different activating agents at their optimum pH. It is difficult to avoid the conclusion that the structure of at least the active site of the enzyme is different when dithionite is the activating agent than when cysteine or 2,3-dimercaptopropanol is used for activation.

As with *Proteus vulgaris*, we cannot reverse by light the CO inhibition of the enzyme from *Desulfovibrio desulfuricans*, activated with the addition of dithionite.

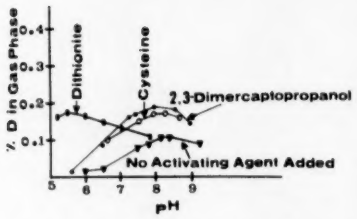

FIG. 2.—Effect of pH on the activity of hydrogenase with different activating agents. Activity was determined by the deuterium exchange method.[5] The D_2O concentration of the liquid phase was 20%, temp. 35°C. The pH values are the final pH values of the reaction mixture after the addition of the activating agents. All buffers were 0.2 *M*.

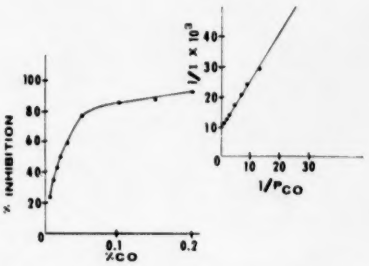

FIG. 3.—Inhibition of hydrogenase of *D. desulfuricans* by carbon monoxide.

Inhibition by CO: The effect of different concentrations of CO in the dark in the absence of activating substances is shown in Figure 3. The inhibition could be reversed by removal of CO and filling the flasks with hydrogen as reported by Purec *et al.*;[6] 0.02 per cent CO caused a 50 per cent inhibition, while with 0.2 per cent CO, the enzyme was more than 90 per cent inhibited.

In insert in Figure 3, the reciprocal of the per cent inhibition of the rate of exchange is plotted against the reciprocal of the partial pressure of CO. This linear relationship is consistent with the assumption that the enzyme combines reversibly with CO,

$$E + CO \rightleftharpoons E{\cdot}CO.$$

This enzyme preparation is more sensitive to CO inhibition than previously reported for *D. desulfuricans*,[6] possibly because in our hands activation by dithionite was not necessary. It has been observed that the hydrogenase of *P. vulgaris* is inhibited at a lower concentration of CO in the absence of dithionite than in its presence.[6]

Reversal of CO inhibition by light: The CO inhibition of hydrogenase activity of an enzyme preparation not activated with addition of a reducing agent was reversed by light. The effect of light intensity on the reversibility of CO inhibition is shown in Table 1. The enzyme was inhibited 84 per cent in the dark. The inhibition decreased as the intensity of illumination was increased.

TABLE 1

EFFECT OF LIGHT INTENSITY ON CO REVERSIBILITY*

	Light intensity (foot candles)	Activity (% D/hr)	Inhibition (%)
H_2	dark	0.264	—
CO-H_2	dark	0.042	84
	5,600	0.050	81
	11,000	0.095	64
	23,000	0.136	48

* A 90-ml quartz flask containing the hydrogenase from *D. desulfuricans* (pH 7.4) was filled with H_2, shaken at 5°C, and the rate of exchange was determined. The flask was evacuated, filled with 0.02% CO-99.98% H_2, and the rate of exchange determined in the dark. The flask was then illuminated with a 2,000-watt bulb. The light intensity was varied by changing the input voltage to the incandescent bulb. The light intensity was determined in foot candles by an exposure meter (Sekonic Electric Co., Japan).

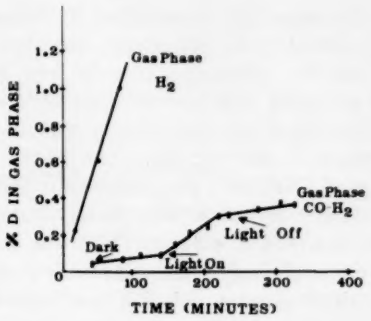

FIG. 4.—Reversal of CO inhibition by light. A 90-ml flask containing dialyzed pH 4 precipitate of *D. desulfuricans* extract, pH 7.4, was filled with hydrogen and shaken at 5°C. The rate of exchange was determined and was 0.78% D/hour. The flask was then evacuated and filled with 0.1% CO-99.9% H_2 and shaken in the dark. It had an exchange rate of 0.032% D/hour (96% inhibition). At 140 min, the flask was illuminated with a 2,000-watt bulb and the rate of exchange increased to 0.153% D/hour (80% inhibition). At 220 min, the light was turned off; the rate of exchange decreased to 0.039% D/hour (95% inhibition).

The effect of alternating periods of dark and light is shown in Figure 4. This experiment was carried out at 5°C in order to increase the extent of photodissociation. The activity of the CO-inhibited system increases with illumination and decreases in the dark, indicating that CO compound of hydrogenase is decomposed by light. Similar results were obtained with a high-pressure mercury arc, though prolonged exposure seems to gradually inactivate the enzyme.

With this enzyme system the activation by light takes place with a lag period of less than 10 min, while with the hydrogenase of *P. vulgaris*,[6] it is 30–60 min and once the illumination is shut off, the system reverts to the inhibited state. The hydrogenase of *D. desulfuricans* differs in several respects from the enzyme in *P. vulgaris*; it has a greatly different susceptibility to CO inhibition and the light reversal has a different kinetic behavior.

Reactivity of hydrogenase with sulfhydryl reagents: The inhibitory effect of reagents which can react with sulfhydryl groups is shown in Table 2. The in-

TABLE 2

EFFECT OF SULFHYDRYL REAGENTS ON HYDROGENASE ACTIVITY AND REVERSIBILITY OF INHIBITION

Inhibitor	Inhibitor concentration (M)	Inhibition (%)
Iodoacetate	6×10^{-2}	30
	12×10^{-2}	51
	20×10^{-2}	98
Iodoacetamide	1.2×10^{-2}	32
	4×10^{-2}	71
	6×10^{-2}	94
N-Ethyl maleimide	1.6×10^{-3}	21
	4.0×10^{-3}	42
	2×10^{-2}	95
p-Chloromercuribenzoate	7×10^{-5}	70
	14×10^{-5}	90
	21×10^{-5}	100
PCMB + cysteine (20×10^{-3} M)	30×10^{-5}	5–10
PCMB + glutathione (20×10^{-3} M)	30×10^{-5}	5–10

The enzyme activity was determined by the deuterium exchange method, pH 8.0, temperature 35°C.

hibition of the enzyme activity by p-chloromercuribenzoate was completely reversed by addition of excess cysteine or glutathione. The effect of the inhibitors was the same whether the enzyme was preincubated with the inhibitor aerobically or added after incubation with hydrogen.

The reversal of CO inhibition by light adds further support to the already existing evidence that hydrogenase from *D. desulfuricans* is an iron enzyme.[2, 4] If such

were the case, then the addition to the native enzyme of a metal which complexes more strongly with SH groups than iron should result in the displacement of the iron and the appearance of ionic iron in solution. Silver is such a metal. We have prepared hydrogenase from *D. desulfuricans* by extraction of the acetone-precipitated cells. The extract was dialyzed for 2 days at 4°C against 0.05 *M* phosphate buffer pH 7.2. The fraction precipitating between 50–90 per cent saturated $(NH_4)_2SO_4$ was dissolved in buffer and again dialyzed for 2 days at 4°C. This sample had an activity in the deuterium exchange assay of 3.5 atom per cent excess/hour/ml of extract. Iron was determined with the reagent bathophenanthroline according to the directions of Frederick Smith Chemical Co.[7] To 1 ml of purified enzyme solution was added hydroxylamine hydrochloride, acetate buffer, and bathophenanthroline. The final pH was 4.6. The mixture was extracted into 10 ml of isoamyl alcohol. The optical density was determined at 533 mμ. Controls gave a density of 0.045 for 1 μg iron. The 1 ml of the purified enzyme gave an absorption of 0.009 corresponding to 0.2 μg iron/ml. Addition of 0.5 ml of M-$AgNO_3$ to the enzyme resulted in an increase of ionic iron to 1.1 μg/ml.

Similar results were obtained with a crude dialyzed enzyme preparation.

It is interesting in connection with the phenomena found with photosynthetic pyridine nucleotide reductase by Fry and San Pietro[8] that acidification of a sample of this enzyme to pH 1.8 purified by precipitation by 50–90 per cent $(NH_4)_2SO_4$ and subsequent removal of nucleoproteins by protamine resulted in a positive test for H_2S by the method of Fogo and Popowsky[9] (0.2 μg H_2S/ml enzyme solution).

Summary.—The data offered in this paper are all consistent with the view that the hydrogenase of *D. desulfuricans* is an iron sulfhydryl enzyme; it is inhibited by some compounds known to react with SH groups, it is inhibited by CO and the inhibition reversed by light, and the addition of silver ions results in the liberation of ionic iron. More definitive information depends now on the preparation of a pure enzyme.

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*AN RNA "REPLICASE" INDUCED BY AND SELECTIVE FOR A VIRAL RNA: ISOLATION AND PROPERTIES**

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The existence of the RNA viruses raises obvious questions concerning information transfer in organisms with an RNA genome. Doi and Spiegelman[1] have shown that neither before nor after infection can sequences be detected in the DNA of the host cell which are complementary to the viral RNA. These results suggest that RNA viruses do not employ DNA as an informed intermediary in any synthetic step required for components unique to the formation of virus particles. On this ground and others one is led to predict[2, 3] a mechanism of RNA replication involving an RNA-dependent RNA polymerase, which we will hereafter refer to as a "*replicase*." (We have elsewhere pointed out[2] that the repetitive use of such phrases as "DNA-dependent RNA polymerase," "RNA-dependent RNA polymerase," etc., can be avoided by introducing three simple terms. As noted, the RNA-dependent RNA polymerase which functions to turn out RNA replicas is termed a "*replicase*." The corresponding enzyme which synthesizes DNA duplicates can be called a "duplicase." Finally, the transcribing enzyme which employs DNA as a template to synthesize complementary RNA can be referred to as a "transcriptase." These terms have the advantage of brevity, precision, and a useful alliterative allusion.)

Available evidence makes it unlikely that an enzyme using an RNA template functions in uninfected cells. All recognized cellular RNA components, including the "message" fraction,[4–6] ribosomal,[7, 8] and amino acid transfer RNA[9, 10] have been shown to be complementary to some sequences in homologous DNA. Consequently, the pathway of their formation can be adequately explained by the DNA-dependent RNA synthesizing mechanism. This conclusion is further strengthened by the observations that actinomycin D inhibits[11] normal cellular RNA synthesis without interfering with the appearance of viral RNA.[12]

The following two predictions are generated by the considerations just summarized: (1) the infecting RNA strand of the virus must serve as a message and be conserved during its translation into protein; (2) an enzyme should be obtainable from cells infected with RNA viruses which is uniquely dependent on RNA to exhibit RNA polymerizing activity.

The first prediction was confirmed[13] through the use of double labeling (N^{15} and P^{32}) and the demonstration that the two isotopes could be recovered in the same RNA strands at the end of a complete lytic cycle. It is the purpose of the present paper to offer evidence which confirms the expectation that a new type of polymerase is induced by an RNA virus.

The experiments to be described were performed with the RNA bacteriophage MSϕ2 used in the earlier investigations noted,[1, 13] and is similar to the f2 of Loeb and Zinder.[14]

A search for a unique RNA-dependent polymerase is complicated by the presence of a variety of enzymes[15] which can incorporate ribonucleotides either terminally or subterminally into pre-existent RNA chains. In addition, there are others (e.g.,

RNA phosphorylase,[16] DNA-dependent RNA polymerase,[17] polyadenylate synthetase[18]) which can effect extensive synthesis of polynucleotide chains. Many of these sources of confusion can be avoided by suitable adjustment of the assay conditions and supplementary tests for a requirement of all four triphosphates, etc.

The most serious difficulty is introduced by the DNA transcriptase, since it appears that, when isolated, it can employ certain types of RNA as substitutes for DNA as templates for polyribonucleotide synthesis.[19, 20] Under these circumstances, the use of actinomycin D or DNAase does not ensure against observing its activity. The only certain way to avoid interference with the DNA-dependent enzyme is to eliminate it from the fraction of interest. It is obvious that a claim for a new type of RNA polymerase must be accompanied by evidence for RNA dependence and a demonstration that the enzyme is distinguishable in one or more of its properties from previously known enzymes with which it can be confused.

Several recent reports offer suggestive evidence of polyribonucleotide synthesis activity induced by RNA viruses of animal[21] and bacterial hosts.[22] However, in no case was an enzyme isolated in a sufficiently purified state to permit a definitive demonstration of RNA dependence. Under these circumstances, investigation of template specificity was obviously impossible.

It is the purpose of the present paper to show that an RNA-dependent polymerase can be isolated from *E. coli* cells infected with an RNA bacteriophage (MSϕ2). Further, the enzyme shows a selective preference for its own RNA as a template.

Materials and Methods.—1. *Bacteria and virus:* The bacterial virus, MSϕ2, was provided by Dr. Alvin J. Clark; it was grown and assayed according to the procedures of Loeb and Zinder.[14] Preparation of virus stocks and purified viral RNA followed the methods of Doi and Spiegelman.[13]

2. *Preparation of infected cells:* The medium used contained the following in gm/liter: Bactotryptone, 10; NaCl, 10; Difco yeast extract, 5; glucose, 1; to each liter was added 1.3 ml of 2 *M* $CaCl_2$. The procedure was as follows: (1) one liter of a log phase culture ($O.D._{660}$ of 0.150) is inoculated with phage at multiplicity of 0.1–0.2 and allowed to go into complete lysis and then used as a source of virus inoculation for larger batches. Thirty-five liter quantities of cells are grown up in cyclone pumps to an $O.D._{660}$ of 0.3. They are then infected with a multiplicity of 10 and allowed to aerate for several min. The aeration is interrupted 10 min for absorption, reinstituted, and the infection allowed to proceed 30–40 min. The process is stopped by chilling with crushed ice, the cells are harvested, and then stored frozen at −20°C. Such cells retain their ability to yield active enzyme for periods exceeding 4 months. Uninfected cells are prepared by the same procedure and stored in the same manner.

3. *Labeled substrates:* C^{14}-labeled riboside triphosphates were all purchased from Schwarz BioResearch, Inc. They were used at the following specific activities: ATP-C^{14}, 2×6 cpm/μM; GTP-C^{14}, 0.72×10^6 cpm/μM; UTP-C^{14}, 1.8×10^6 cpm/μM; CTP-C^{14}, 1.7×10^6 cmp/μM. P^{32}-labeled nucleotide was prepared by a modification of Tener's[23] procedure suggested to us by A. M. Michelson. The method is illustrated by the synthesis of UMP^{32}: a mixture of 100 μM of inorganic phosphate and 200 μM of isopropylidine is dissolved in 3 ml of dry pyridine, and the solution evaporated to dryness. More pyridine is added, and the procedure is repeated several times. The residue is finally dissolved in 0.1 ml of dimethylformamide and 1 ml of pyridine, to which is added 200 μM of β-cyanoethanol and then 1,000 μM of dicyclohexylcarbodiimide, and the mixture is left at 37°C overnight under anhydrous conditions. Pyridine is removed under reduced pressure, and to the residue is added 2 ml of 2 *N* NaOH; the mixture is kept at 100°C for 30 min. To remove the protecting isopropylidene residue, the mixture is adjusted to pH 1.0 with 2 *N* HCl, kept at 100°C for 45 min, and then neutralized to pH 8.0. (If applied to purine nucleosides, the acidic hydrolysis should be at pH 2.0 at 100°C for 30 min.) Usually 80–90% conversion of inorganic phosphate into nucleoside-5′-phosphate is achieved. Conversion to the triphosphate is accomplished by the baker's yeast kinase as described by Weiss.[24] The UTP^{32} was employed at 2×10^6 cmp/μM.

4. *Reagents:* Unlabeled riboside triphosphates were from Pabst Laboratories, Milwaukee, Wisconsin. DNAase was 2 × recrystallized from Worthington Biochemical Company, Freehold, New Jersey. It was further purified on DEAE columns to remove contaminating ribonuclease.[25] Phosphoenolpyruvate (PEP) and the corresponding kinase (PEP-kinase) were from C. F. Boehringer and Soehne, Mannheim, Germany. Lysozyme was purchased from Armour and Company, Kankakee, Illinois. Poly A, Poly U, and Poly C were obtained from the Miles Chemical Company, Clifton, New Jersey. Poly G was a gift from Dr. J. Fresco, and turnip yellow mosaic virus (TYMV) RNA was kindly provided by Dr. R. Haselkorn. Tobacco mosaic virus RNA was isolated and purified from infected plants supplied by Dr. L. M. Black.

5. *Assay of enzyme activity by incorporation of radioactive nucleotides:* The standard reaction of 0.25 ml contained the following in μM: Tris-HCl pH 7.5, 21; $MgCl_2$, 1.4; $MnCl_2$, 1.0; KCl, 3.75; mercaptoethanol, 0.65; spermine, 2.5; phosphoenolpyruvate, 1.0; $(NH_4)_2SO_4$, 70; CTP, ATP, GTP, and UTP, 0.5 each. In addition, it contained pyruvate kinase, 5 μg, DNAase, 2.5 μg, and, where indicated, 10 μg of the polynucleotide being tested as template. Enzyme was assayed at levels of 50–300 μg protein per sample. DNAase was always omitted in assaying for DNA-dependent polymerase activity. Incubations were carried out at 35°C for 10 min and terminated by placing the reaction mixture in an ice bath and by the addition of 0.15 ml of neutralized saturated pyrophosphate, 0.15 ml of neutralized orthophosphate, and 0.1 ml of 80% trichloracetic acid (TCA). The precipitate was washed onto a millipore filter and washed five times with 10 ml of cold 10% TCA containing 0.9% of Na pyrophosphate. The millipore membrane was then dried and counted in a liquid scintillation counter, as described previously.[4] The pyrophosphate was included in the wash, since we found in agreement with Kammen *et al.*[26] that its presence lowered zero time backgrounds to acceptable levels (40–70 cpm per sample containing input counts of 1×10^5 cmp).

6. *Preparation of enzyme:* A suitable aliquot of the frozen infected cells is removed and put through the following procedure which is specified for 2 liters of infected cells at 1×10^9 cells per ml. (*a*) The frozen cells are suspended in "standard buffer" (0.01 *M* Tris, pH 7.2; 0.005 *M* $MgCl_2$; 0.0005 *M* mercaptoethanol) and allowed to thaw. To this, 5 μg/ml of DNAase and 1 mg/ml lysozyme are added, and the mixture is frozen and thawed twice, as described by Hayashi and Spiegelman.[4] The resulting mixture is allowed to incubate for 10 min at 20°C after increasing the level of DNAase to 10 μg/ml. (*b*) The extract is centrifuged at 15,000 *g* for 20 min, and the supernate and pellet are separated. The pellet is frozen with dry-ice acetone and ground in prechilled (−15°C) mortar for 10 min. It is then resuspended in 5 ml of "standard buffer" and again centrifuged at 15,000 *g* for 20 min. The two supernates are combined and possess an $O.D._{260}$ of about 300. (*c*) The crude supernate is made up of 25 ml with "standard buffer" and adjusted to 0.01 *M* EDTA and incubated at 0°C for 5–15 min. The appearance of a light, white precipitate indicates the onset of ribosomal destruction. The extract is then centrifuged at 15,000 *g* for 20 min and the precipitate discarded. (*d*) The extract is now subjected to a protamine fractionation which is designed to remove the DNA-dependent RNA polymerase, while leaving the RNA-dependent enzyme in the supernatant. The amount of protamine required to remove virtually all of the DNA-dependent activity varies from 11–13 mg per ml and must be titrated by assay for DNA-dependent activity. After appearance of the precipitate, the extract is centrifuged at 10,000 *g* for 10 min. (*e*) The supernate of the protamine fraction is kept, and to 40 ml is added 23.6 ml of a saturated ammonium sulfate (pH adjusted to 7). After 10 min the material is centrifuged at 10,000 *g* for 10 min. (*f*) The activity is found in the supernate, and to it is added a further 12 ml of saturated ammonium sulfate followed by centrifugation at 10,000 *g* for 10 min. (*g*) The supernate is discarded; the precipitate is dissolved in 6 ml of "standard buffer" and dialyzed against 1 liter of the same buffer for 2 hr. (*h*) The dialysate is then put on a DEAE cellulose column (1.2 × 7 cm) and washed with 40–100 ml of the "standard buffer" made 0.1 *M* with respect to sodium chloride. This effectively removes remaining protamine sulfate and contaminating nucleases. Following the 0.1 *M* wash, the enzyme is eluted with 20 ml of the "standard buffer" made 0.3 *M* with respect to sodium chloride. (*i*) To 20 ml of the eluted enzyme is added 20 ml of a saturated ammonium sulfate, and the enzyme is centrifuged down at 10,000 *g* for 10 min. (*j*) The precipitate is dissolved in 4 ml of the "standar l buffer."

The usual preparation obtained from the above procedure contains 7.4 mg protein per ml

with an O.D.^{280}/O.D.^{260} ratio of 1.2. The enzyme activity is unstable unless the ionic strength is restored. The addition of $(NH_4)_2SO_4$ to 10% of saturation was found to prevent detectable decay of activity for several weeks providing the enzyme is stored in a 0°C ice bath.

Results.—Reasons for including certain components in our "standard assay mixture" (see *Methods*, § 5) may be specifically noted. The riboside triphosphate generating system (PEP and PEP-kinase) was routinely included to avoid contributions by RNA-phosphorylase to the observed incorporation. DNAase was added to eliminate DNA-dependent synthesis. Spermine had previously been found[27] to be an effective neutralizer of ribonuclease commonly found in crude extracts.

From our first examinations of extracts from infected cells, evidence was obtained for DNAase-resistant incorporating activity associated with the pellet fraction which was obtained at 100,000 *g* in 2 hr (100G120P). This feature is illustrated in the data of Table 1 in which the pellet contains a fair amount of DNA-dependent activity of which 16 per cent continues in the presence of DNAase. If the pellet fraction is treated with EDTA as in *Methods*, § 6(*c*) and the supernatant then fractionated with protamine as in *Methods*, § 6(*d*), activities exhibited are as shown in Table 2. Prior to the protamine fractionation there is considerable DNA-dependent activity. The protamine step effectively removes this, the ratio of DNA- to RNA-dependent activity changing from 23.3 to 0.09.

It was found with experience that separation of the extract into the 100G120P pellet and supernatant was unnecessary, and the procedure described in *Methods* § 6 was adopted for routine purposes. Table 3 shows some of the general characteristics of the enzyme fraction thus obtained. It has a clear requirement for viral RNA, and synthesis is only observed when all four triphosphates are present. The omission of any one of them completely abolished incorporation of UMP^{32}. It should also be noted (from the last line of Table 3) that this preparation does not contain detectable amounts of the polyadenylate synthetase.

TABLE 1

LOCATION OF ENZYME ACTIVITY IN INFECTED CELL EXTRACTS

Fraction	Template	NT incorporated in mμM/10 min/mg protein
Pellet	MSφ2-RNA	0.34
	0	0.28
	CT-DNA*	1.94
Supernatant	MSφ2-RNA	<0.02
	0	<0.02

* DNAase omitted from assay mixture.
Assay made with UTP^{32} under the "standard conditions" as described in *Methods*, § 5. All template polynucleotides added to a level of 10 μg per reaction mixture.

TABLE 2

SEPARATION OF RNA-DEPENDENT FROM DNA-DEPENDENT POLYMERASE

Fractions	Template	NT incorporated in mμM/10 min/mg protein
(A) Supernatant of EDTA treated 100G120P	0	0.14
	MSφ2-RNA	0.54
	CT-DNA*	12.6
(B) Prot. sulfate (12 mg/ml) supernatant of A	0	0.18
	φ2-RNA	3.5
	CT-DNA*	0.31

* DNAase was omitted from assay mixture.
Assays were carried out with UTP^{32} under conditions of *Methods*, §5. See text for details on fractions.

TABLE 3

TEMPLATE AND TRIPHOSPHATE DEPENDENCE OF ENZYME

Assay mixture	Template	NT incorporated in mμM/10 min/mg protein
(1) Complete	0	0.12
(2) "	MSφ2-RNA	4.73
(3) −ATP	" "	<0.02
(4) −GTP	" "	<0.02
(5) −CTP	" "	<0.02
(6) *−GTP, CTP, UTP, i.e., C^{14}-ATP only	" "	0.08

* C^{14}-ATP was present as the only triphosphate.
Enzyme was carried through all the steps described in *Methods*, §5. It had an O.D.^{280}/O.D.^{260} ratio of 1.21. UTP^{32} incorporation assayed according to *Methods*, §6.

TABLE 4

NEAREST NEIGHBOR ANALYSIS OF PRODUCT SYNTHESIZED BY RNA POLYMERASE UNDER INFLUENCE OF VIRAL RNA

Per cent mole fractions			
$Cp^{32}U$	$Ap^{32}U$	$Up^{32}U$	$Gp^{32}U$
25.5	25.1	16.3	33.1

0.8 ml of the "standard reaction mixture" (*Methods*, §5) containing UTP^{32} as the labeled riboside triphosphate was incubated for 15 min. The reaction was stopped with 0.3 ml of neutralized saturated Na pyrophosphates and 7 ml of cold 3.5% perchloric acid (PCA). The precipitate was washed 5 times with 7 ml of 3.5% PCA. At each washing 0.8 mg of *E. coli* ribosomal RNA was added as carrier. The final precipitate was dissolved in H_2O and the perchloric acid removed by addition of 0.5 ml *N* KOH and centrifugation. The supernate was incubated at 37°C for 18 hr, and the nucleotides were separated and analyzed on Dowex-1 formate.[8]

It was necessary to demonstrate that the UMP^{32} was incorporated into the internucleotide linkages of a heteropolymer. A larger-scale reaction was run, the product hydrolyzed with alkali, and a nearest neighbor to U determined. The results (Table 4) provide clear evidence that a proper heteropolymer is being synthesized.

A very interesting feature was revealed when the template specificity of the enzyme preparation was examined (Table 5). It will be noted that there is a

TABLE 5

TEMPLATE SPECIFICITY OF PURIFIED RNA-DEPENDENT POLYMERASE*

Template (all at 10 γ/0.25 ml)	NT incorporated in mμM/10 min/mg protein
0	0.08
MSφ2-RNA	8.5
s-RNA	0.09
Ribosomal RNA	0.06
Ribosomal RNA + MSφ2-RNA	8.0
TMV-RNA	0.3
TYMV-RNA	2.2
CT-DNA†	0.11

* UTP^{32} incorporation assayed according to *Methods*, §5.
† DNAase omitted from assay mixture.

striking preference for its own RNA. There is little detectable activity with either the host s-RNA or ribosomal RNA. Further, ribosomal RNA does not interfere with the template activity of the MSφ2-RNA. Of the two plant viruses tested, TMV exhibits low but probably significant activity. TYMV-RNA definitely serves as a template with an efficiency which routinely corresponds to 25 per cent of that observed with MSφ2-RNA.

The enzyme preparations used in the experiments of Tables 4 and 5 contained very little of the DNA-dependent RNA polymerase activity. Consequently, the properties shown cannot be ascribed to contamination by the transcriptase. Nevertheless, as a final check a purified DNA transcriptase was prepared from *E. coli*

according to the procedure of Chamberlin and Berg,[28] and it had a specific activity with DNA of 6,000/mg protein in their units. When presented with MSϕ2-RNA as a template, it showed virtually no activity. Finally, it should be noted that many preparations were made from noninfected cells according to the procedure described in *Methods* (§ 6). None of these showed the specific stimulation by MSϕ2-RNA nor the requirement of all four riboside triphosphates shown in Tables 3 and 5 for the same preparations derived from virus-infected cells.

Discussion.—The experiments described demonstrate that cells infected with an RNA virus contain an RNA polymerase possessing a number of characteristics which serve to identify it as a unique entity. When sufficiently pure, it needs an RNA template for activity. The fact that DNA will not substitute in this requirement clearly distinguishes it from the DNA-primed polymerase. When presented with a heteropolymer containing all four bases, the enzyme has an absolute requirement for all four riboside triphosphates. This characteristic, coupled with the direct demonstration of inactivity with ATP alone (line 6, Table 3), clearly eliminates the polyadenylate synthetase of August *et al.*[18] These same characteristics also serve to distinguish this enzyme from RNA-phosphorylase.

An outstanding feature of this enzyme is its preference for its homologous RNA as a template for activity. The fact that the ribosomal RNA and s-RNA molecules of the host cell are virtually completely inactive as templates is perhaps not too surprising. The production of an enzyme which ignores the mass of pre-existent cellular RNA represents an obvious advantage to the virus. Replica production can thus be focused on the single strand of viral RNA which is the ultimate origin of the final yield of progeny.

It is to be noted that the polymerase induced by MSϕ2 can readily distinguish its own RNA from that of TMV, and even to some extent that of TYMV. This selectivity differentiates this enzyme from other sorts of RNA synthetases. It also may serve as a unique label to permit identification among the class of polymerases induced by the RNA viruses. One is inclined to predict, for example, that the RNA polymerase induced by TMV-RNA will show an equally one-sided preference for its own RNA as a template. It seems likely that the basis of this sort of specificity will be found in the sequences of the RNA, but the details remain to be unraveled.

It will be recalled that with only one possible exception[29] all RNA viruses are single-stranded. Further, no evidence for an RNA-replicating duplex, analogous to that of the single-stranded DNA virus, ϕX174,[30,31] has thus far appeared, despite the fact that RNA-RNA duplexes are known to be very stable structures and resistant to ribonuclease.[32] These observations raise the obvious possibility[13] that RNA replication may not mimic in all details the mechanism of DNA duplication. However, now that the RNA "replicase" has been obtained free of RNA it should be possible to perform the experiments necessary for an understanding of its mode of action.

Summary.—An RNA polymerase has been isolated and purified from cells infected with an RNA virus. It shows a dependence on RNA for polymerizing activity and a mandatory requirement for all four riboside triphosphates when employing a template which contains all four bases. The enzyme exhibits a selective preference for its homologous RNA, being completely inactive with host

s-RNA and ribosomal RNA. It is partially functional with certain other viral RNA's.

We would like to express our deep appreciation to Dr. A. M. Michelson who during a stay at Illinois introduced us to the intricacies of nucleotide chemistry. The synthesis of labeled intermediates was thereby converted from a terrifying adventure in uncertainty to a pleasant chore.

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*GLUCOCORTICOID STIMULATION OF THE BIOSYNTHESIS OF GLUTAMIC-ALANINE TRANSAMINASE**

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Among the mechanisms which have been considered as possible descriptions of the molecular events underlying hormone action, one which has attracted increasing attention among contemporary biochemists is the proposal that control of the biosynthesis of specific enzymes may be an important factor (see, e.g., ref. 1). The plethora of enzyme activities which are found to increase or decrease in response to an alteration in endocrine state in the whole animal[2] has been an insistent factor in favor of this view. More sophisticated studies have come forth in recent years which have given further encouragement to this concept. These have been of three types, of which the following are examples. It has been demonstrated that there is an increased amino acid incorporation into carbamyl phosphate synthetase of tadpole liver after exposure of the animals to thyroxine[3] and into a partially purified preparation of glutamic-tyrosine transaminase of rat liver after exposure of the animals to cortisol.[4] Early hormonal effects have been found on RNA metabolism,[5, 6] particularly of the nuclear fraction.[7, 8] Finally, altered hormonal states have a striking influence on amino acid incorporation into total protein in cell-free systems.[9–15] We are presenting in this paper what we believe to be unequivocal evidence that a change in endocrine state leads to a change in the rate of biosynthesis of a specific enzyme.

Materials and Methods.—The definition of activity units and methods of enzyme assay and antibody titration of rat liver glutamic-alanine transaminase have been presented previously.[16, 17] The procedure for purification of the enzyme to a homogeneous state and some of its physical and chemical properties will be published elsewhere.[18]

Results.—In order to interpret meaningfully the results of measurements of the influence of hormones on amino acid incorporation into a specific protein, it is necessary to know that the period of exposure to the labeled amino acid is short relative to the half-life of the enzyme. Otherwise, the enzyme may have become fully labeled during the experimental period under all conditions. This information is lacking in the studies with carbamyl phosphate synthetase.[3] The half-life of a protein can be measured by isotopic methods if it can be isolated, or more simply by enzyme activity measurements, if a method is available for perturbing its steady-state level in tissues. The latter possibility is readily realized with enzymes, the activity levels of which in tissues are susceptible to hormonal alteration, once it has been established that the activity level is a valid measure of the amount of catalytically active protein present, as in the case at hand.[17]

The prednisolone-mediated transformation from the normal steady-state level to what may be called the high glucocorticoid steady-state level is shown in Figure 1. The high hormone steady-state level is virtually achieved in 5 days, as previously reported by Rosen *et al.*[19] On the eighth day, at the point marked by arrows on the figure, hormone administration was discontinued, and the activity allowed to return

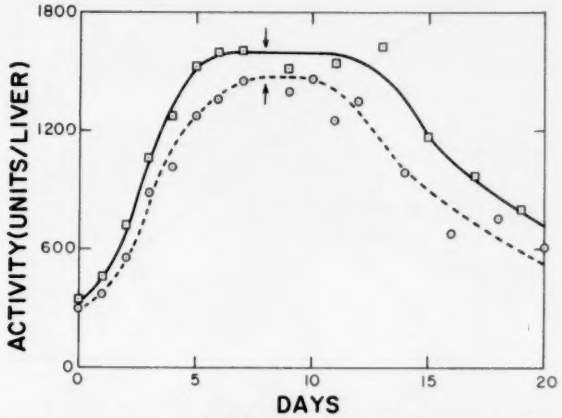

FIG. 1.—Effect of administration of prednisolone and subsequent withdrawal on glutamic-alanine transaminase activity of rat liver. In the upper curve (squares), animals were injected subcutaneously with 4 mg/day of prednisolone acetate, and in the lower curve (circles), 2 mg/day. Each point represents the mean of 3, or occasionally 2, 200–250-gm male rats. Injections were in the morning, and for the first 7 days the animals were sacrificed 2 hr after the last injection. At the points marked with the arrows, the administration of hormone was discontinued.

to the normal steady-state level. Thus, the first part of the curve represents the effects in animals under the influence of a high hormone level, and the second part represents animals under the influence of a normal or lower hormone level. It can be seen from inspection of the curves that the rise in enzyme activity to the steady-state value characteristic of the high hormone level is more rapid than the fall to the normal steady-state level. This is shown quantitatively in the log plots in Figures 2 and 3 (see below).

For the calculation of half-lives, it was assumed that the rate of change of enzyme level was the algebraic sum of a zero-order rate of synthesis and a first-order rate of breakdown (Table 1). It is not assumed that the rate constants for either synthesis

TABLE 1

ENZYME LEVEL AS A FUNCTION OF TIME

	Normal	Prednisolone
	$\frac{d(E)}{dt} = k_1 - k_2(E)$	$\frac{d(E)}{dt} = k_1' - k_2'(E)$
At steady state:	$k_1 = k_2(E_0)$	$k_1' = k_2'(E_0')$
At any time t:	$\ln [(E) - (E_0)] = \ln [(E_0') - (E_0)] - k_2 t$	$\ln [(E_0') - (E)] = \ln [E_0') - (E_0)] - k_2' t$

The unprimed terms are the values in normal animals and the primed terms the corresponding value in the prednisolone-treated animals.

or breakdown are the same in the normal and the high hormone animals. A plot of $\ln [(E) - (E_0)]$ versus t as the activity is returning to the normal steady-state level allows the determination of k_2, the rate constant for the degradation reaction under normal hormone conditions (Fig. 2); and a plot of $\ln [(E_0') - (E)]$ versus t as the activity is rising to the steady-state value of the high hormone level allows the cal-

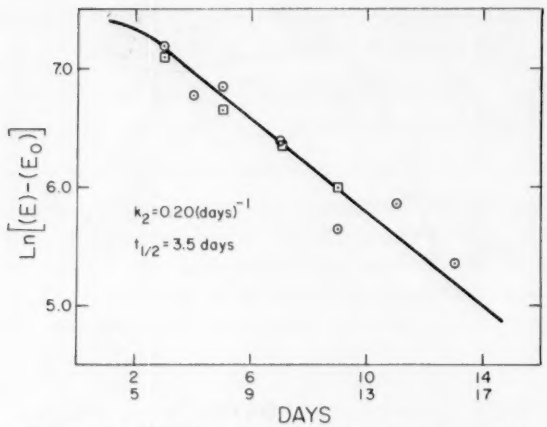

FIG. 2.—Logarithmic plot of the decline of enzyme activity after withdrawal of hormone. Symbols as in Fig. 1. The abscissa is days after withdrawal of hormone—the upper set of numbers for the experiment with 2 mg/day of prednisolone and the lower set for the experiment with 4 mg/day. If allowance is made for the greater lag period in the latter experiment, the data from the two experiments lie on the same line.

culation of k_2', the rate constant for the degradation reaction under conditions of high hormone (Fig. 3 and Table 1). The rate constant for degradation of the enzyme is about 3 times as great when the animal is under the influence of a high hormone level compared with the normal, and the half-life is therefore $^1/_3$ as great. These data appear to indicate that the administration of the hormone stimulated the rate of breakdown of the enzyme some threefold. It has been reported that glucocorticoid administration also increased the rate of turnover of serum albumin in the rabbit and man.[20] The half-lives thus obtained for glutamic-alanine transaminase may be compared with values for other rat liver proteins of about 3 hr for glutamic-tyrosine transaminase,[4, 21] 3 hr for tryptophan pyrrolase,[22] 1 day for catalase,[23] 5 days for arginase,[24] and 3 days for total liver protein.[24] Significantly in muscle, where levels of enzyme activities are notably refractory to environmental influences, the half-life of aldolase was 20 days in the rat.[25]

From the rate constants for degradation and the steady-state levels of the enzyme, it is possible to calculate the rates of synthesis of the enzyme in the normal and prednisolone-treated animals (Table 2).

TABLE 2

CALCULATED RATES OF GLUTAMIC-ALANINE TRANSAMINASE SYNTHESIS IN NORMAL AND PREDNISOLONE-TREATED RAT LIVERS

Status	(E_0) (units/gm liver)	k_2 $(\text{days})^{-1}$	Calculated k_1 (units/gm liver) × $(\text{days})^{-1}$	Calculated k_1 (mg/gm liver) × $(\text{days})^{-1}$
N	31.4	0.20	6.3	0.021
P	108	0.60	65.4	0.22

N refers to normal animals and P refers to animals injected with 2 mg of prednisolone acetate per day. (E_0) is the steady-state level of enzyme. k_2 is the experimental value for the rate constants of enzyme degradation (Figs. 2 and 3). $k_1 = k_2(E_0)$ (see Table 1). The conversion of units of enzyme to mg of enzyme is based on a specific activity of 300 for virtually homogeneous preparations of the enzyme (ref. 18).

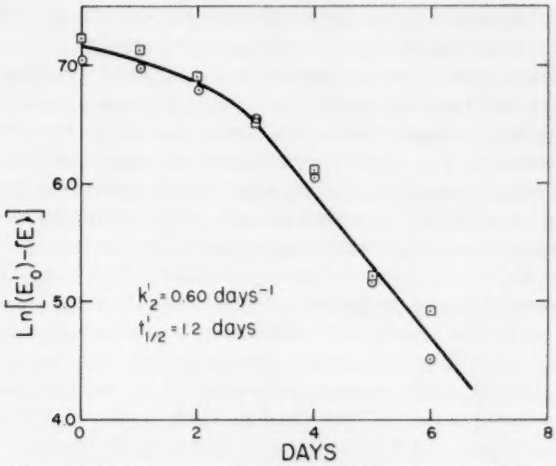

FIG. 3.—Logarithmic plot of increase in enzyme activity consequent to administration of hormone. Symbols as in Fig. 1.

The conclusion from these experiments is that the increase in glutamic-alanine transaminase level after glucocorticoid administration is not an effect of a decreased rate of breakdown; in fact the rate of breakdown is increased in the hormone-treated animals, and must therefore be a result of an increased rate of synthesis. Direct evidence to support this conclusion was obtained by measurements of the relative extent of incorporation of C^{14}-labeled leucine into the enzyme in normal and prednisolone-treated rats. The results are presented in Table 3.

As can be seen, there was a markedly greater incorporation of isotope into the enzyme from glucocorticoid-treated animals, by a factor of about fivefold. Since the half-life of the enzyme is of the order of days, these incorporation data during a 1-hr period can be taken as approximations of the relative rates of synthesis of the enzyme in the two cases, unless the specific activity of leucine in the precursor pools is drastically different. In order to estimate a possible effect of the hormone on the leucine pool specific activity, counts incorporated into the total soluble protein of the liver were also measured. As shown in the table, there was only a minor effect of

TABLE 3

RADIOACTIVE LEUCINE INCORPORATION IN LIVER GLUTAMIC-ALANINE TRANSAMINASE IN NORMAL AND PREDNISOLONE-TREATED RATS

Status	Enzyme activity (units)	Cpm in enzyme*	Cpm in soluble protein	Soluble protein (mg)
N (5)	31.4 ± 1.1	21.0 ± 2.6	14,200 ± 1,400	8.3 ± 0.3
P (7)	108 ± 5.9	99 ± 6.4	18,500 ± 1,900	8.5 ± 0.1
P/N	3.44	4.71	1.3	1.0

* Although there was some variation from animal to animal in the counts incorporated into the enzyme, the ratio of this value to the counts incorporated into total soluble protein (as a measure of relative pool specific activity) was strikingly constant, especially in the normal animals. This indicates that the rate of synthesis of the enzyme from one animal to another is quite uniform.

5.8–13.8 × 10^6 cpm of uniformly labeled C^{14}-L-leucine (specific activity, *ca.* 1 mc/mg) was injected, and the livers were removed 1 hr later, homogenized in 0.25 *M* sucrose, and centrifuged at 100,000 × *g* for 1 hr. The enzyme was partially purified by heat and acid treatment and ammonium sulfate precipitation (ref. 16), then precipitated with antibody (ref. 17). Cpm were corrected for self-absorption and normalized to 5 × 10^6 cpm injected. Numbers in parentheses under "Status" refer to the number of animals in the group. Prednisolone treatment was 2 mg/day for 5 days. P/N is the ratio of the measured values in the hormone-treated relative to the untreated animals. Values are means for 1 gm of liver with standard errors. Protein was determined by the Lowry method (ref. 26).

the hormone on this parameter, of the order expected from the data of Noall *et al.*[27] on the effect of glucocorticoids on α-aminobutyrate transport. Thus, the specific activity of the leucine pool does not seem to be a significantly variable factor in the comparison of the incorporation rates of normal and hormone-treated animals.

Although the peak of *enzyme level* is not achieved until about the fifth day of prednisolone administration (Fig. 1), the increasing *rate of enzyme synthesis* would be expected to reach a maximum much sooner, then remain constant as long as the high hormone level is maintained. Correspondingly, after withdrawal of the hormone the *rate of enzyme synthesis* would presumably diminish to the normal level, followed by a later return of the *enzyme level* to the normal value. The curves in Figures 1, 2, and 3 are consistent with this prediction. In each case there is a lag of the order of a day or more before the logarithmic relationship is established, indicating that a new and constant rate of synthesis and degradation has been achieved. To test this conclusion directly, measurements were made of the relative rates of enzyme synthesis by the isotopic method described in Table 3 after 1 day and 3 days of hormone administration, and 2 days after withdrawal of the hormone. The results are presented in Table 4. Again, the rate of amino acid incorporation in the hormone-treated animals was 4–6 times that in the normals. In harmony with the prediction from theory, the rate of synthesis of the enzyme has reached the new steady-state value considerably earlier than has the enzyme level. This is particularly noticeable in the 1-day-treated animals where enzyme level is only slightly above normal while the rate of synthesis is already maximal. Two days after hormone withdrawal following a 5-day treatment, the rate of synthesis is still high, consistent with the long lag period preceding the achievement of the normal rates of enzyme synthesis and breakdown (Fig. 3). This may be a reflection of the persistence of high hormone levels in the animal.

TABLE 4

RADIOACTIVE LEUCINE INCORPORATION INTO GLUTAMIC-ALANINE TRANSAMINASE AT VARIOUS TIMES AFTER PREDNISOLONE TREATMENT

Days of hormone treatment	Enzyme activity (units)	Cpm in enzyme*	Cpm in soluble protein
0 (6)	35.0 ± 2.1	45.0 ± 9.8	18,700 ± 2,800
1 (3)	59.0 ± 8.5	298 ± 51	23,600 ± 1,500
3 (3)	117 ± 4.1	203 ± 13.3	23,700 ± 1,300
5† (6)	150 ± 12.1	189 ± 21.5	20,500 ± 3,100

* The same constancy in the ratio of counts incorporated into enzyme to counts incorporated into total soluble protein in individual animals was observed here that is referred to in the footnote in Table 3. In these experiments, performed several months after those in Table 3, the counts incorporated into the enzyme were in all cases about double the corresponding values in Table 3. The reason for this difference is not known.

† This group was injected with radioactive leucine and sacrificed 2 days after discontinuance of hormone treatment.

Livers were removed 1 hr after administration of C^{14}-leucine, homogenized in 0.25 *M* sucrose, and centrifuged at 100,000 × *g* for 1 hr. The enzyme was partially purified and precipitated with antibody. Cpm were corrected for self-absorption and normalized to 5×10^6 cpm injected. Values are means for 1 gm of liver with standard errors. Numbers in parentheses under "Days of hormone treatment" are the numbers of animals in each group. There were no significant differences in the total soluble protein content in the several groups.

Discussion.—Three general modes of action have been visualized in considering the molecular events underlying hormone effects, viz., interaction with transport barriers, direct modification of enzyme activity, and control of the synthesis and/or degradation of specific enzymes. The first has been established in connection with the effect of insulin on glucose transport[28] and the second as a factor in the actions of glucagon and epinephrine.[29] Other examples could be cited; however, in these we have referred to, there seems to be a clear relationship between the observed events

on the molecular level and the metabolic effects of the hormone. We believe the results reported here establish the hormonal control of the biosynthesis of a specific enzyme. In this case, however, there does not appear to be a connection between the observed biochemical effect and at least the early metabolic response to glucocorticoid administration. Increased incorporation of alanine and other precursors into glycogen can be shown within 2 hr after prednisolone administration,[30] far too early to be ascribed to changes in glutamic-alanine transaminase activity. On the other hand, the possibility that changes in the activity of this enzyme are responsible for later metabolic responses to glucocorticoids remains a possibility. In any event it seems safe to predict that other metabolic control functions of hormones will be found to be directly related to modifications of the rate of synthesis or breakdown of specific enzymes.

The experiments reported here have not touched on the question of the specific site in the machinery of protein synthesis where hormonal control is exerted. One might speculate, on the basis of the lag preceding the achievement of the maximum rate of enzyme synthesis consequent to hormone administration, that the hormone is acting to stimulate the production of an essential component of the system (messenger RNA) rather than to activate preexisting components. It is hoped that studies on the biosynthesis of the enzyme in cell-free systems will provide further information in this regard.

Summary.—Measurements of C^{14}-leucine incorporation into liver glutamic-alanine transaminase in normal and prednisolone-treated rats has provided a direct demonstration of a severalfold greater rate of biosynthesis of the enzyme in the hormone-treated animals. The elevated rate of synthesis precedes and leads to the increase in the steady-state tissue level of the enzyme consequent to hormone administration. The half-life of the enzyme has been determined and found to be 3.5 days in normal animals and 1.2 days in prednisolone-treated animals.

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*AN INFLUENCE OF INSULIN ON THE SYNTHESIS OF A RAPIDLY LABELED RNA BY ISOLATED RAT DIAPHRAGM**

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Insulin, when added *in vitro* to isolated rat diaphragm, will enhance the incorporation of labeled amino acids[1–4] and of amino acid precursors[5] into protein; neither the precise mechanism nor the exact site at which the hormone acts to bring about this effect is known. The influence of insulin on protein biosynthesis in muscle does seem, however, to be separate and distinct from the action of the hormone in facilitating the entry of glucose into the cell.[3, 4] Moreover, there are observations[5–8] that, at least by indirection, support the hypothesis that insulin stimulates protein biosynthesis through an effect on an intracellular process.

To obtain information as to the identity of the intracellular process, the effect of insulin on nucleic acid synthesis was investigated. Insulin was found to enhance RNA synthesis in muscle;[9, 10] this was reflected in an increase in incorporation of C^{14} from several substrates (adenine, orotic acid, and glucose) into RNA and by an actual net synthesis of RNA. Most remarkably, the latter occurred in the absence of any added substrate.

It is a commonplace that considerable importance attaches to the isolation of the primary biochemical locus at which insulin influences metabolic processes, for that information is vital to an analysis of the mechanism of the hormone's action. Especially pertinent is the identification of the exact molecular species of RNA whose synthesis is increased by insulin. The stimulation of the production of a specific RNA, more so if that RNA were limiting for protein synthesis, as may be the case with messenger RNA,[11] would add weight to the speculation that RNA synthesis is the intracellular site of action of insulin in stimulating protein synthesis.

The recognition of the problem and its implication has led to an attempt to identify the fraction of the cellular RNA whose synthesis is stimulated by insulin. It is the purpose of this paper to report the results of that investigation.

Materials and Methods.—Nonfasted male Wistar or Sprague-Dawley rats, maintained under standard conditions[4] until they reached 110–130 gm, were killed by decapitation and the hemidiaphragms rapidly removed and prepared for incubation. The two hemidiaphragms from a single rat were distributed between control and experimental groups; six hemidiaphragms were incubated in a 50-ml conical flask with 5 ml of Krebs-Henseleit bicarbonate medium (pH 7.4) containing the additions indicated in the figures. Incubation was at 37° in a Dubnoff shaker (120 cycles/min); the gas phase was O_2-CO_2 (95:5).

The adenine-8-C^{14} (specific activity 5.06 mc/millimole) and the carrier-free P^{32}-orthophosphate were purchased from Nuclear-Chicago Corporation or the Radiochemical Centre, Amersham. The insulin (beef zinc insulin crystals, lot 719106) assaying 25.2 units/mg and containing less than 0.1% glucagon was a gift of Dr. O. Behrens of Eli Lilly and Company; it was dissolved in N/300 hydrochloric acid to form a stock solution of 20 units/ml.

Extraction of RNA: After incubation the flasks were drained, and the hemidiaphragms were washed three times with 50 ml of cold 0.05 *M* Tris buffer, pH 7.5, to remove excess isotope. The hemidiaphragms were then placed in an iced mortar; 1 ml of 10% sodium lauryl sulfate and 0.5 ml of bentonite (suspended in 0.05 *M* Tris, pH 8.1) were added immediately and the diaphragm muscle minced and ground to a slurry with addition of 10 ml of 0.05 *M* Tris buffer, pH 7.5. The slurry was transferred to glass-stoppered tubes and shaken at 20° for 8 min; 10 ml of freshly distilled water-saturated phenol was added and shaking continued for 10 min, now at 0°. The preparation was centrifuged (3,500 rpm at 0°) for 15 min and the water layer removed and retained. Water (10 ml) was added to the phenol residue which was shaken for 5 min at 0°, recentrifuged, and the water layer removed. The combined water layers were re-extracted with 10 ml of phenol. To the final water extract was added 1/9 volume of 20% sodium acetate and 3 volumes of 96% ethanol. The RNA was precipitated overnight at −15° and collected by centrifugation at 10,000 rpm for 10 min at 0°. The precipitate was dissolved in 0.4 ml of buffer (0.1 *M* NaCl; 0.01 *M* acetic acid, pH 5.2); the material not dissolved in the buffer was removed by centrifugation and discarded. The RNA was precipitated twice more with ethanol, being dissolved each time in buffer, the material not dissolved in buffer being removed and discarded.

Analysis of RNA: The RNA (0.1 ml) was layered on 4.7 ml of linear sucrose gradient (20–5%) containing 0.1 *M* NaCl and 0.01 *M* acetic acid, pH 5.2. After centrifugation at 37,000 rpm for 4 hr in the SW 39 rotor of a Spinco model L ultracentrifuge, a hole was pierced in the bottom of the tube and drops collected sequentially. The extinction at 260 mμ was determined for each fraction (after addition of 1 ml of water), and their radioactivity assessed after precipitation and washing with ice-cold 0.5 *N* perchloric acid onto membrane filters.

A sample of RNA from diaphragm muscle incubated with P^{32} was washed twice with ice-cold perchloric acid (0.5 *N*) to remove nucleotides, hydrolyzed in 0.1 *N* KOH at 37° for 24 hr, neutralized with perchloric acid, and the nucleotides separated by electrophoresis[12] at pH 3.5 in 0.05 *M* ammonium formate buffer. The nucleotides were identified on the paper under ultraviolet light, the spots cut out, and the nucleotides eluted onto aluminum planchets, dried, and counted in a gas flow counter. Each of the four nucleotides contained P^{32}, indicating the labeling of the RNA was not due merely to terminal addition of radioactive nucleotides.

Adenine-8-C^{14} uptake: Six hemidiaphragms were incubated in 20 ml of Krebs-Henseleit bicarbonate medium containing 1 μc/ml of adenine-8-C^{14} and with or without insulin (0.1 unit/ml) for 2 hr at 37°. At the end of the incubation, the hemidiaphragms were rapidly weighed and then minced and ground to a slurry in ice-cold 10% trichloroacetic acid. The trichloroacetic acid-soluble supernatant was collected by centrifugation; the precipitate was washed twice with ice-cold 10% trichloroacetic acid, and the washes were retained. A 1.5-ml sample of the pooled soluble extract was transferred to a stainless steel planchet, evaporated to dryness, and counted. A 0.1-ml sample of the incubation medium was diluted to 5 ml; 1 ml was transferred to a planchet, dried, and counted in a like manner. The results are expressed, in the first instance, as the ratio of the number of counts per ml of diaphragm water (calculated on the assumption that the diaphragm tissue contains 75% water) divided by the number of counts per unit volume remaining in the incubation medium.

Uptake was reckoned as the difference between the radioactivity originally present in the medium and that remaining at the end of incubation. The results are expressed as counts per min per gm of tissue for 2 hr.

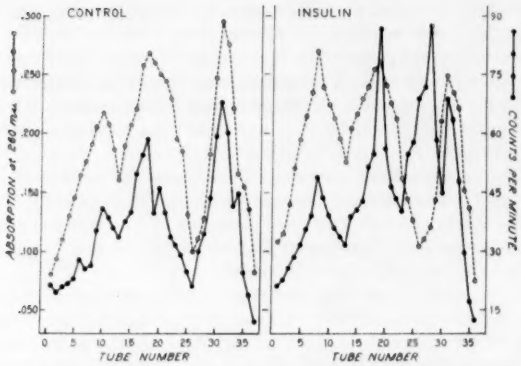

FIG. 1.—Sedimentation of C^{14}-adenine-labeled RNA from rat diaphragm muscle: effect of insulin. Six paired hemidiaphragms were incubated for 2 hr at 37° in 5 ml of Krebs-Henseleit bicarbonate buffer containing 1 μc/ml of adenine-8-C^{14} and with or without insulin (0.1 unit/ml); no glucose was added. At the end of the incubation the RNA was extracted and analyzed on linear sucrose gradients as described in *Materials and Methods*.

Samples of the acid-soluble tissue extract were evaporated to dryness and the residue redissolved in 1 ml of 1 *N* HCl. The nucleotides were separated by ascending paper chromatography[13] on Whatman No. 3 filter paper in methanol-ethanol-HCl-H_2O (50:25:6:19). The bases were located on the paper with an ultraviolet light and a radiochromatogram scanner. The adenine spot was cut out and eluted with 1.5 ml of 0.1 *N* HCl, the absorption determined at 260 and 280 mμ, and the molar concentration calculated; 1 ml of the eluate was plated, dried, and counted. The specific activity of the adenine in the intracellular pool was then calculated.

Results.—The results were conditioned by several parameters, but especially by the duration of the experiment, the nature of the radioactive substrate, and whether the tissue was exposed continuously to the substrate or a pulse of radioactivity was employed. But no matter the experimental conditions, three extinction peaks were obtained with sedimentation coefficients of 4, 19, and 28 S (Figs. 1–3). The first is probably transfer RNA while the other two are of ribosomal origin. This finding is constant and is uninfluenced by insulin. The specific radioactivity of the three species of RNA is conditioned by the duration of incubation, the transfer RNA being labeled first and the ribonucleoprotein RNA later (experiments not shown); usually, but by no means always, the 19 and 28 S peaks have similar radioactivity.

In the first experiment (Fig. 1), diaphragm was incubated with adenine-8-C^{14} (1 μc/ml) and with or without insulin (0.1 unit/ml) for 2 hr; no glucose was added. The fractionation on sucrose gradients of RNA from muscle in the control experiment (no insulin) revealed the transfer RNA (4 S) to be most radioactive, the other two fractions to be less so. Analysis of RNA from insulin-treated diaphragms disclosed the following: The specific activity of at least one species of ribonucleoprotein RNA (19 S) was increased by the hormone. But perhaps the most remarkable finding was the appearance of a new peak of radioactivity of extremely high specific activity (i.e., high counts, low extinction) and of a sedimentation constant (between 4 and 18 S) that suggests it is messenger RNA. The latter finding, however,

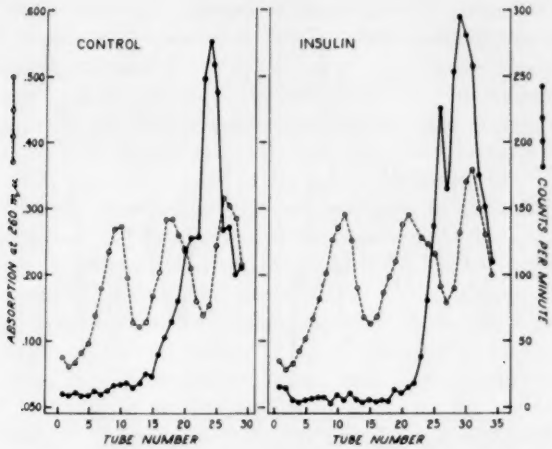

FIG. 2.—Sedimentation of P^{32}-labeled RNA from rat diaphragm muscle: effect of insulin. Six paired hemidiaphragms were incubated for 5 min at 37° in 5 ml of Krebs-Henseleit bicarbonate buffer (from which KH_2PO_4 was omitted) containing 0.5 mc of P^{32}-orthophosphate and with or without insulin (0.1 unit/ml); no glucose was added. The RNA was prepared and analyzed as described in *Materials and Methods*. Note that only 28 drops were collected in the control experiment, whereas 35 were obtained in the insulin experiment.

has not been entirely constant. Although this fraction is not seen in experiments of this design without added hormone, it is not uniformly present in experiments with insulin.

In the next experiment (Fig. 2), diaphragm was incubated for only 5 min with 0.5 mc of P^{32} and with or without insulin (0.1 unit/ml); no glucose was added. Most of the radioactivity was in the peak that precedes transfer RNA; this rapidly labeled RNA fraction has an S value similar to that described for messenger RNA of *E. coli*.[14] The amount of rapidly labeled RNA was increased by insulin.

When diaphragm is incubated with P^{32} for a longer period, i.e., 2 hr (Fig. 3), one no longer sees a peak corresponding to messenger RNA (which is presumably buried in the other peaks) and notes instead that insulin increases the specific activity of ribonucleoprotein RNA as in the instance of the experiment with C^{14}-adenine (cf. Fig. 1).

One interpretation of the results is that insulin increases the rate and magnitude of transport into the muscle cell interior of the RNA precursors. If that were so, then the RNA isolated would be more radioactive, even if there were no change in the rate of RNA synthesis. This possibility must be given credence since insulin increases the transport of a number of substrates[15] and, in fact, there are recorded preliminary observations[16] that insulin accelerates phosphate uptake by muscle, an observation of great pertinence for the present experiments. This being the case, we felt it necessary to determine if insulin altered adenine-8-C^{14} uptake by isolated diaphragm and, more to the point, if the hormone changes the specific activity of the intracellular adenine pool. Neither parameter was influenced by insulin

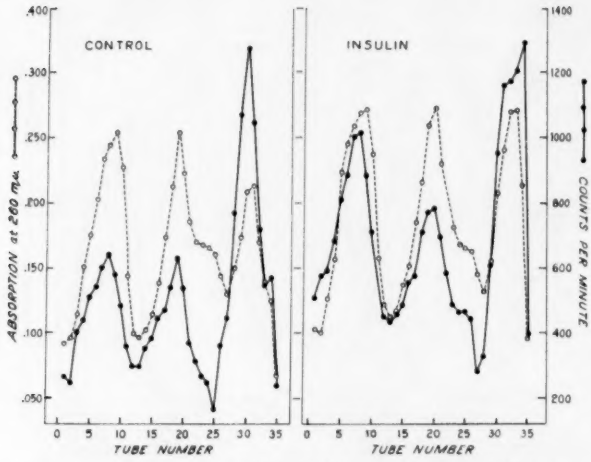

FIG. 3.—Sedimentation of P^{32}-labeled RNA from rat diaphragm: effect of insulin. Conditions of incubation and analysis of RNA were the same as in Fig. 2 except that incubation was for 2 hr rather than 5 min.

(Table 1), making it most unlikely that insulin accelerates RNA synthesis by an effect on substrate transport. It is noteworthy, however, that insulin increases the utilization of adenine-8-C^{14} (Table 1) and, inasmuch as it does so without changing the ratio of the concentration of the base in the cell interior to that in the extracellular space (incubation medium), and without changing the specific activity of the intracellular adenine pool, this may be taken as being in accord with a direct stimulatory effect of insulin on RNA synthesis.

Discussion.—A number of factors complicate the interpretation of experiments of the type recorded here. First, in the absence of added insulin, muscle does synthesize protein; since it is the rate of synthesis that appears to be accelerated by the hormone, one would expect all the components of the synthesizing machinery, including messenger RNA, to be present. One would predict an effect of insulin on the rate of a particular reaction rather than on the appearance of some new reaction. This accords then with the observation that there is synthesized in muscle a rapidly labeled nucleic acid, having the characteristics of messenger RNA, and that the synthesis of this fraction is in some circumstances increased by insulin (cf. Fig. 2). However, it is also true that in other instances insulin appears to

TABLE 1

UPTAKE AND ACCUMULATION OF ADENINE-8-C^{14} BY ISOLATED RAT DIAPHRAGM AND SPECIFIC ACTIVITY OF INTRACELLULAR ADENINE: INFLUENCE OF INSULIN

	Accumulation cpm/ml of tissue water / cpm/ml of medium	Uptake cpm/gm of tissue/2 hr	Specific activity intracellular adenine cpm/$10^{-5}M$
Control	0.035 ± 0.004	57,100	5,650 ± 423
Insulin (0.1 unit/ml)	0.037 ± 0.002	118,600	4,960 ± 632

Six hemidiaphragms were incubated in 20 ml of Krebs-Henseleit bicarbonate medium containing 1 μc/ml of adenine-8-C^{14} and with or without insulin (0.1 unit/ml) for 2 hr at 37°; no glucose was added. The analyses were made as described in *Materials and Methods*. The values for adenine utilization are from a single observation done in triplicate; the values for accumulation and specific activity are the mean ± S.E. of six separate observations.

be crucial in conditioning the appearance or persistence of messenger RNA (cf. Fig. 1). It is no less significant that insulin consistently increases the specific activity of ribonucleoprotein RNA.

This brings one to a second point, namely, that for mammalian cells there is no assurance that the RNA that is rapidly synthesized in the nucleus serves exactly the same role as it does in bacteria. The absence of this assurance considerably restricts analysis of the significance of the effect of insulin on RNA synthesis. Despite this restraint, the finding that insulin leads, in some experiments and under some conditions, to an increase in the synthesis of messenger RNA and an increase in the specific activity of ribosomal RNA has led to the postulation that RNA synthesis is the molecular site of action of insulin in promoting protein synthesis and that perhaps the same action accounts for the other biochemical and physiological effects of the hormone.[17, 18] The theory and its implications are discussed *in extenso* in another place,[17,18] but, briefly, it is proposed that insulin coordinates the synthesis of protein and other molecules by initiating the transcription of a series of messages, perhaps, by directly combining with a repressor molecule, or, alternatively, by leading to the formation of a specific metabolite that combines with the repressor. By a mechanism of this sort, insulin might initiate an increase in the rate of synthesis of a series of specific proteins, which proteins (enzymes) might condition the several anabolic actions of the hormone.

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POLYRIBOSOME BREAKDOWN AND HEMOGLOBIN SYNTHESIS

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Recent reports have shown that polyribosomes, structures containing several ribosomes probably held together by messenger RNA, are the major sites of protein synthesis in recticulocytes,[1-3] in rat liver,[4] in HeLa cells,[5] and in *E. coli*.[6] Also, these structures may be formed when poly U is added to *E. coli* ribosomes.[6-8] We have previously reported briefly on the breakdown of polyribosomes coincident with hemoglobin synthesis. In these earlier studies, 80S ribosomes were also active for hemoglobin synthesis, but joined the aggregates during synthesis and left on completion of a globin chain.[9] Here we report the orderly breakdown of polyribosomes dependent on hemoglobin synthesis, along with evidence that each ribosome is liberated from the polyribosome structure as it completes and releases a globin chain. Also, evidence for formation of polyribosomes by attachment of 80S ribosomes is presented. A mechanism involving ribosome movement along the messenger RNA coordinated with peptide-chain synthesis was postulated by Gierer,[3] Warner *et al.*,[10] and Gilbert.[6] A similar conclusion was reached by Noll *et al.*[11] using liver ribosomes.

Materials and Methods.—Reticulocyte ribosomes were isolated as described by Allen and Schweet.[12] Preparations designated 1X microsomes were centrifuged once and suspended by gentle hand homogenization with a loose-fitting lucite homogenizer in either solution D (10^{-2} *M* Tris buffer, 10^{-3} *M* KCl, 10^{-4} *M* $MgCl_2$), or 0.25 *M* sucrose in distilled water. Thus, 1X microsomes were pelleted only once before use, whereas 2X microsomes were resuspended in medium B[12] and precipitated by centrifugation a second time before use.

Usually, fresh whole supernatant solution from ribosome preparations was used for the preparation of soluble enzyme fractions. Two types of preparations designated "regular enzyme" and "purified enzyme" were used. Regular enzyme was prepared by making whole supernatant[12] to 0.1 *M* Tris HCl at pH 7.5. Powdered ammonium sulfate was then slowly added with stirring to give 40% saturation. After 30 min the mixture was centrifuged, the precipitate was discarded, and powdered ammonium sulfate added to the supernatant to give 70% saturation. After 30 min with gentle stirring, the precipitate was collected by centrifugation and then dissolved in 0.1 *M* Tris-HCl buffer at pH 7.5 and dialyzed for 18 hr against 2 changes of 0.02 *M* Tris-HCl buffer, pH 7.5 containing 10^{-3} *M* GSH and 10^{-4} *M* EDTA. The resulting "regular enzyme fraction" contained sRNA and was relatively rich in hemoglobin even though most of the hemoglobin present in the whole supernatant solution was not precipitated with ammonium sulfate at 70% of saturation.

The preparation of "purified enzyme" differed from that of regular enzyme in that a 1.0% solution of protamine sulfate in 0.1 *M* Tris HCl at pH 7.5 was slowly added with stirring to the whole supernatant to give a final concentration of protamine sulfate of 0.34 mg/ml. After 30 min, the resulting protamine-RNA precipitate was removed by centrifugation. Ammonium sulfate was then slowly added with stirring to the supernatant as above. After 30 min with stirring, the precipitate was removed by centrifugation, discarded, and ammonium sulfate added to the supernatant as before to give a 70% saturated solution. The precipitate was dissolved in a solution containing 0.1 *M* Tris chloride at pH 7.5, 5×10^{-4} *M* GSH, and 10^{-4} *M* EDTA, and brought to a volume equal to one-half the volume of the whole supernatant originally used. The pH of the solution was adjusted to 7.0 by the cautious addition of 1.0 *M* acetic acid, and the fraction which precipitated between 40–70% saturation with ammonium sulfate prepared as before. The precipitate was dissolved in the same volume of 0.1 *M* Tris buffer containing GSH and EDTA as used before. The pH of the resulting solution was cautiously adjusted to 6.4 with 0.1 *M* acetic acid and ammonium sulfate added to give 70% saturation. The precipitate was collected by cen-

trifugation, dissolved in 0.002 *M* Tris HCl, pH 7.5 containing 10^{-3} *M* GSH plus 10^{-4} *M* EDTA to give 1/30 the volume of the whole supernatant used. This solution was then dialyzed against 2 changes of the same solution for 18 hr. The resulting "purified enzyme fraction" contained less than 0.6% nucleic acid by optical density measurements and was only light pink due to contaminating hemoglobin.

Assay procedure: The complete reaction mixture used was that previously described,[12] with minor variations. The complete reaction mixture contained 4.0 mg of ribosomes except for those tubes analyzed for ribosomal composition in the analytical ultracentrifuge or applied to sucrose gradients, in which cases 6.0 mg of ribosomes and 1–2 mg of purified enzyme or 12 mg of regular enzyme plus the other usual components were employed. The mixtures were incubated at 37°C, then either precipitated with 5% TCA in the presence of about 10 mg of casein per tube, or chilled in ice for analysis in the analytical ultracentrifuge or on sucrose gradients. All samples were taken through the complete washing procedure.[12] Radioactivity was determined using either a Nuclear-Chicago gas flow counter or a Packard Tri-Carb scintillation counter. C^{14}-valine incorporation × 8.3 was used to calculate the mμ moles of total amino acids incorporated into hemoglobin per mg of ribosomes (ribonucleoprotein). For poly-U-induced polyphenylalanine synthesis, the amount of phenylalanine incorporated into hemoglobin was subtracted from the total polyphenylalanine synthesized.

Analysis of ribosome composition: The composition of reticulocyte ribosomes was determined from schlieren images obtained during sedimentation near 0° in a Spinco Model E analytical ultracentrifuge simultaneously using a regular and a wedge window cell. Unless otherwise specified, ribosomes were analyzed at a concentration of 4.25 mg/ml with a bar angle of 40° in the regular complete system incubation mixture. Controls run in 0.01 *M* KCl plus 10^{-4} *M* $MgCl_2$ gave similar patterns. In some cases, reaction mixtures contained relatively large amounts of hemoglobin which made them opaque to light of a wavelength shorter than about 600 mμ, thus necessitating an optical system utilizing red light. This was accomplished by using a Wratten no. 29 red filter with the standard high-pressure mercury arc of the analytical ultracentrifuge and Kodak Type 1-N or Type 103-F spectroscopic plates, either of which gave satisfactory sensitivity in the red region.

Relative concentrations of the ribosomal components were measured by determining the area under the peaks of the schlieren profiles. Appropriate schlieren images were enlarged by projection to about 8 × 10 in. on single weight Kodak Kodabromide F-5 photographic paper. A base line was fitted to the enlarged profile on the resulting photograph, then each peak was carefully cut out and weighed. After correction for radial dilution within the centrifuge cell, the weights of the photographic paper representing each peak were directly proportional to the concentration of the species of ribosomes present in the sample. This method was found to give greater accuracy than could be obtained with a planimeter. Despite such errors as occur in fitting a base line to the schlieren patterns and determining the outer edges of the peaks, the method gave values that were reproducible within a few per cent, and were in good agreement with percentage compositions of ribosomes determined by sucrose gradient centrifugation.

Sucrose gradients: Linear sucrose gradients were formed for a Spinco SW 25.1 swinging bucket rotor using 7.5–30% w/v recrystallized sucrose (Fisher reagent grade) in solution D. Gradients were formed at room temperature, then the tubes were allowed to stand 30–60 min in ice before 1.0 ml of cold complete reaction mixture containing 4.25 mg of ribosomes was layered on each gradient. These were centrifuged at 24,000 rpm for 4 hr at a refrigeration setting of the centrifuge that maintained the temperature near 0° throughout the run.

Ten-drop fractions were collected from each tube. An aliquot of each fraction was removed and diluted for determination of the optical density at 260 mμ; then casein and 5% TCA were added to the remaining portion of each fraction. All fractions were then washed and counted as previously described. Since drop size (and therefore fraction size) was found to vary, data was corrected to and plotted on the basis of 30 fractions per gradient tube. Both optical density and incorporation are expressed as values for 1.0-ml fractions.

Results.—Polyribosome breakdown and protein synthesis: When 1X ribosomes (see *Methods*) were incubated in the complete system and the mixtures cooled to 4° after various incubation times, a progressive breakdown of polyribosomes was

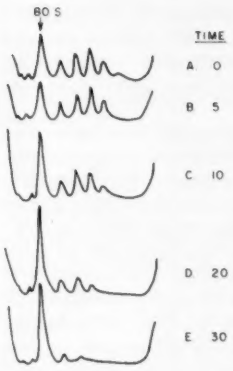

FIG. 1.—Sedimentation patterns of reticulocyte ribosomes after incubation in the cell-free system. Ribosome preparations were incubated in the complete system for hemoglobin synthesis for the indicated number of min. Aliquots were cooled to 0° and analyzed in the analytical ultracentrifuge (see *Methods*). The direction of sedimentation is from left to right. Centrifugation was generally about eight min at 42,000 rpm at 0°.

observed (Fig. 1). The peaks seen in these patterns have sedimentation constants of approximately 190, 170, 150, 130, 110, and 80S, confirming earlier reports.[1-3] The percentages of the various sizes of polyribosomes and 80S particles in the 1X ribosomes are similar to those in an unpelleted extract (unpublished data).

Three points of interest can be seen in Figure 1. The breakdown is essentially a conversion of polyribosomes to 80S ribosomes which is completed in the same time period that amino acid incorporation occurs. The heavier polyribosomes break down first (compare Figs. 1*B* and 1*C*). There is a lag period in the breakdown during the earlier time periods even though amino acid incorporation is linear during this time (compare Fig. 1*A* to 1*B*, with 1*B* to 1*C*). Further documentation of these points is given below.

A quantitative analysis of polyribosome breakdown is shown in Table 1. The percentage of the various sizes of polyribosomes and 80S particles varied to some extent in various preparations. For example, some preparations contained up to 40 per cent of 80S particles. This probably reflects small differences in the handling procedure and in the analysis of the analytical ultracentrifuge patterns (see *Methods*). However, a zero time control of the same preparation was usually run for comparison. The striking dependence of polyribosome breakdown on the presence of energy is evident (Table 1). The polyribosomes remaining after 20 min of synthesis in this experiment (Table 1) reflect the slow incorporation of this experiment, as will be shown below. Puromycin at a concentration which inhibited hemoglobin synthesis by 90 per cent did not inhibit polyribosome breakdown (Table 1). However, this is consistent with evidence that puromycin inhibition does not prevent peptide-bond formation, but results in the premature release of small peptides.[13, 14]

Polyribosome breakdown was also dependent on, and correlated with, protein synthesis when this was varied by changing the enzyme preparation or omitting the amino acid mixture (Table 2). The proportionality between the amount of protein synthesis and amount of polyribosome breakdown with various enzyme fractions was good in a 20-min assay. The absence of amino acids decreased incorporation more than it did breakdown. In other experiments, the omission of amino

TABLE 1

BREAKDOWN OF 1X POLYRIBOSOMES

Incubation	Per cent Composition					
	80S	110S	130S	150S	170S	190S
Incubated at 4°	31.8	10.4	14.3	20.0	15.2	5.1
Complete system	63.4	8.2	17.2	8.0	...	...
Complete, minus energy	32.6	12.5	15.2	18.6	14.4	5.7
Complete, plus puromycin	70.4	10.3	11.5	7.6	...	...

The samples were incubated in the complete amino acid incorporating system for 20 min at 37° (except for the control). In "No energy," the ATP, GTP, creatine phosphate, and creatine kinase were omitted. Puromycin was added at a concentration of 7×10^{-4} *M*. The dots indicate no detectable material at this position.

acids had less effect on the rate of protein synthesis, and a more direct correlation with breakdown could be obtained. In addition, there is an early lag in polyribosome breakdown which has not been considered here (see below). All of these results taken together demonstrate that polyribosome breakdown is a function of protein synthesis.

If the cause of the breakdown were the detachment of 80S ribosomes from the messenger as they completed synthesis of a chain, there should be an orderly shift in polyribosome sizes during protein synthesis toward the smaller sizes and a proportional increase in the 80S ribosomes. As shown in Figure 2, this is the case. The heaviest polyribosomes disappear first (190S), followed in order by the other sizes. With longer incubation times, or with more rapid protein synthesis, more complete disappearance of all polyribosomes was observed. There is a small amount of nonspecific breakdown, but the polyribosomes present initially are converted to 80S ribosomes in about 85 per cent yield. Secondly, as shown previously,[9] the 80S ribosomes are not labeled; that is, the formation of 80S ribosomes was accompanied by the release of the completed chain (Fig. 3). The large increase in labeled hemoglobin occurred between 5 and 20 min, during which period the number of 80S ribosomes increased greatly. During this time, the labeled polysomes maintained a similar steady-state radioactivity or decreased slightly, although further incubation which resulted in complete polyribosome breakdown led to a decrease in radioactivity. In general, the final radioactivity in the polysome peak was 10–15 per cent of that in released hemoglobin.[9]

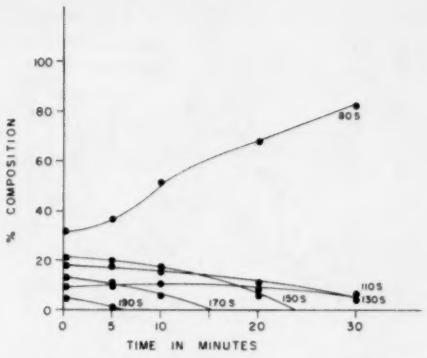

FIG. 2.—Breakdown pattern of reticulocyte ribosomes. Ribosome preparations were incubated as described for Fig. 1, and the quantitative analysis of several runs were averaged. The points show the percentage of the total of each size of ribosome at various times.

We have previously noted that 80S ribosomes were active for hemoglobin synthesis and aggregated onto the polyribosome if these were present.[9] Re-formation of polyribosomes is also indicated by the lag in polyribosome breakdown and formation of 80S particles shown in Figure 2. Polyribosome breakdown did not occur

TABLE 2

POLYRIBOSOME BREAKDOWN AND PROTEIN SYNTHESIS

Incubation conditions	Per cent polyribosomes	Per cent breakdown	Incorporation	
			mμmoles amino acids	Per cent of total
Zero time	62.5	0	0	0
Purified enzyme	8.0	87.2	9.8	92.5
Regular enzyme	22.0	65.0	7.0	66.0
No enzyme	48.8	21.5	1.6	15.0
Minus amino acids	29.4	53	2.9	27.5

The samples as described were incubated in the complete system for 20 min, then chilled and aliquots taken for ultracentrifuge analysis and determination of radioactivity. The final total incorporation of 10.6 mμmoles of total amino acid was reached in 25 min with the purified enzyme, and 60 min with the regular enzyme. The latter was slower than usual. The % polyribosomes was the sum of all sizes greater than 80S.

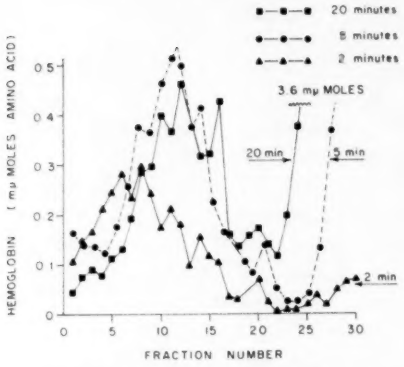

FIG. 3.—Labeling pattern of reticulocyte ribosomes. 1X ribosomes were incubated in the complete system for the indicated times and then analyzed on a sucrose gradient. The data are given as mμmoles of total amino acid incorporated per tube. The optical density patterns corresponding to these times would be similar to the results shown in Fig. 1. 80S ribosomes show a peak at tube 20. The bottom of the gradient (heavier polyribosomes) is to the left.

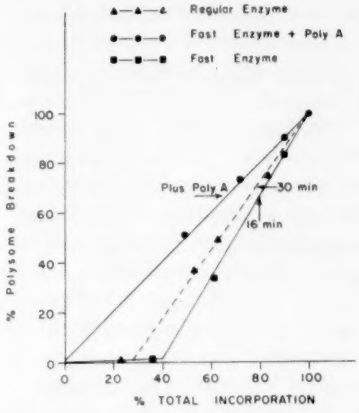

FIG. 4.—Rate of polyribosome breakdown and protein synthesis. The samples were incubated for various periods in the complete system, and aliquots used for determination of radioactivity and for analysis in the analytical ultracentrifuge. The final incorporation at the completion of the reaction was the 100% value (usually 40 min of incubation). The initial amount of all ribosomes larger than 80S was considered the 100% polysome value.

until 30–40 per cent of the total incorporation had taken place and then was proportional to the amount of protein synthesis (Fig. 4). When different enzymes or amounts of enzyme were used, the final amount of synthesis and polyribosome breakdown were similar, but different lengths of time were required. Thus, 80 per cent of the total incorporation gave about 70 per cent polyribosom ebreakdown, although this required 16 and 30 min, respectively, with different enzyme preparations (Fig. 4). Since incorporation and release of completed chains do not show a lag, re-formation of polyribosomes during the early stages of synthesis could explain the initial delay in polyribosome breakdown (see *Discussion*).

Effects of synthetic polynucleotides: The inhibition of hemoglobin synthesis by poly U has been reported.[15] Inhibition of hemoglobin synthesis by poly U and by poly A is shown in Figure 5. The inhibition of hemoglobin synthesis by poly U was 60 per cent of maximal at levels of poly U where no polyphenylalanine formation took place. Poly A was also inhibitory, although thus far, polylysine synthesis has not been reported in this system. In addition, the maximal inhibition by poly U and poly A was about 65 and 40 per cent, respectively, even at very high levels of these polymers. Despite the inhibition, polyribosome breakdown was accelerated by the addition of poly A, but only when added to the complete system (Fig. 6). The same orderly breakdown was noted (cf. Figs. 2 and 6). No breakdown occurred in the presence of poly A if energy was omitted. Similar results, in general, except for the preservation of 110S and 130S particles, were found with poly U and will be reported in detail.[16] These facts suggested that poly A might inhibit hemoglobin synthesis by preventing attachment of 80S ribosomes to polyribosomes.

This would permit the polyribosomes to complete and release globin chains and 80S ribosomes, but would prevent the starting of new chains and re-formation of polyribosomes. This mechanism for the inhibition caused by poly A is supported by the data in Figures 4 and 6. It can be seen that the more rapid breakdown of polyribosomes in the presence of poly A was due largely to the absence of a lag phase. This was shown most strikingly when polyribosome breakdown was plotted against incorporation (Fig. 4).

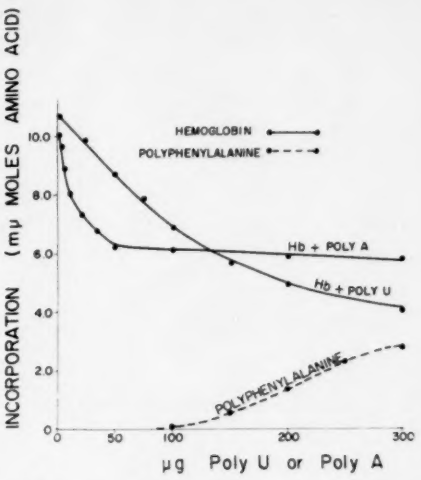

FIG 5.—Effect of poly A and poly U on incorporation. The data are given as mμmoles of total amino acids or polyphenylalanine per mg ribosomes. The synthetic polynucleotides were added in the indicated amounts to the complete system (1.4 ml) at zero time, and standard assay conditions were used.

Discussion.—The polyribosome structure in reticulocytes breaks down only during protein synthesis in the cell-free system. The breakdown depends on the amount of protein synthesis and on the presence of energy, enzyme, and amino acid mixture. The fact that this can be observed in the analytical ultracentrifuge or on sucrose density gradients implies that a large proportion of the ribosomes in the polyribosome structure are active for protein synthesis. However, the data do not exclude the possibility that some of the ribosomes in these structures are inactive, but remain attached in some way until protein synthesis causes the structure to break down. During protein synthesis the largest polyribosome clusters disappear first, followed by the next largest, and so on. It should be noted that the rate of breakdown depends on the polyribosome size and not on the per cent composition. Such an orderly breakdown as a result of protein synthesis suggests a mechanism involving growth of the polypeptide chain as the 80S ribosomes move along the polyribosome structure, and release of single 80S ribosomes from the end of the cluster upon completion of the polypeptide chain. The association of labeled polypeptide chains with smaller polyribosomes as incubation in the cell-free system continues (Fig. 3) is also consistent with this mechanism. A similar breakdown of liver polyribosomes during protein synthesis has been reported by Noll.[11]

For this mechanism to be valid, it is important to demonstrate that the detachment of 80S ribosomes from the polyribosome is related to the *completion* of the polypeptide chain. This was not possible in earlier studies where the nascent chains remained attached to the 80S ribosomes.[6, 11] In the reticulocyte cell-free system, completed globin chains are released, and this was associated with polyribosome breakdown and formation of unlabeled 80S ribosomes (Fig. 3). The synthesis which accompanies the breakdown of polyribosomes should result only in the completion of already started chains. The N-terminal region of such chains therefore would not be labeled during incubation in the cell-free system. Attachment of 80S

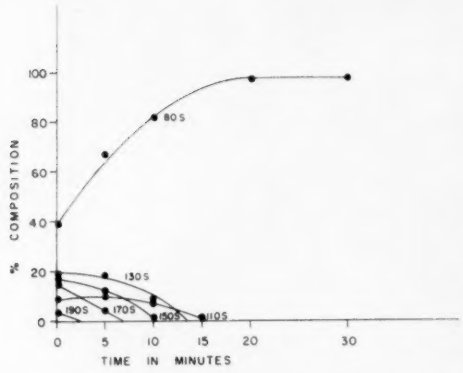

FIG. 6.—Breakdown patterns of reticulocyte ribosomes in the presence of poly A. Conditions are the same as in Fig. 2, except for the addition of 100 μg of poly A.

ribosomes to polyribosomes in the cell-free system would be required for complete *de novo* synthesis of globin chains. Earlier, we reported the *de novo* synthesis of globin chains in the cell-free system.[9] However, 80S ribosomes were active, and it was indicated that they could attach to polyribosomes. Attachment of 80S ribosomes in the present studies is suggested by the lag in polyribosome breakdown at early time periods. The peculiar inhibition of hemoglobin synthesis by poly A, which does not exceed 40 per cent inhibition and which accelerates polyribosome breakdown, can be explained if poly A acts to prevent attachment of 80S ribosomes to polyribosomes.

These conclusions have been tested directly[17] by following hemoglobin formation with ribosomes labeled in the intact cell with H^3-leucine and incubated in the cell-free system with C^{14}-leucine, by measuring the appearance of N-terminal C^{14}-valine (a measure of *de novo* synthesis), and by studying reattachment of P^{32}-labeled 80S ribosomes to polyribosomes. The results of these experiments support the mechanism of hemoglobin synthesis proposed here.

Since polyribosomes do not break down during protein synthesis in the intact cell for at least 60 min (unpublished data), it appears that the limiting factor in the cell-free system is the reattachment of 80S ribosomes to the polyribosome structure.

Summary.—The breakdown of polyribosomes in the reticulocyte cell-free system occurred only during protein synthesis and was accompanied by the release of completed chains. The largest sizes of polyribosomes disappeared first, followed in order by the other sizes. Inhibition of hemoglobin synthesis by poly A resulted in an increased rate of orderly breakdown. Indirect evidence for reattachment of 80S ribosomes to polyribosomes was presented. These results support a mechanism of protein synthesis, involving attachment of 80S ribosomes to the polyribosome which initiates the formation of the peptide chain, followed by chain growth and finally detachment of the ribosome and release of the completed polypeptide chain.

Note added in proof: After this manuscript was completed, evidence for this type of mechanism of protein synthesis in HeLa cells was reported by Goodman, H. M., and A. Rich, *Nature*, **199**, 318 (1963).

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*ROLE OF SERINE IN CHYMOTRYPSIN ACTION. CONVERSION OF THE ACTIVE SERINE TO DEHYDROALANINE**

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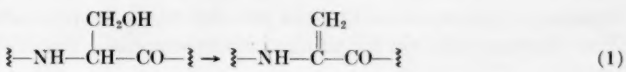
Serine is apparently *at* or *near* the active center of many enzymes. It is the residue that is phosphorylated or acylated by inhibitors and substrates in such enzymes as chymotrypsin,[1] trypsin,[1] thrombin,[2] elastase,[3] subtilisin,[4] cholinesterase,[5] phosphoglucomutase,[6] and alkaline phosphatase.[7] Although powerful arguments can be presented[8, 9] that it is indeed the key nucleophilic residue in the catalytic action of these enzymes, its role remains a source of controversy.

The reasons for this controversy are threefold. The first stems from the fact that essentially all of the "serine enzymes" have hydroxylic substrates, e.g., glucose and water. These acceptors have essentially the same reactivity as serine, and hence it is difficult to conceive of a catalytic role for a residue having the same properties as the final acceptor. The second objection stems from kinetic evidence[10–12] which is difficult to reconcile with an intermediate step involving serine; instead it suggests, or apparently requires, a direct attack by the water or glucose acceptor. Finally, all the reported modifications of serine which render an enzyme inactive involve replacing the H of the hydroxyl with a bulkier group. Hence, it can be argued that such serine modification prevents enzyme action because access to the true catalytic group, e.g., histidine, is blocked rather than because any intrinsic property of serine is changed.

To evaluate these arguments, a chymotrypsin in which the "active serine" residue was converted into a dehydroalanine residue (cf. equation 1) seemed highly desirable.



(1)

The conversion of the $-CH_2OH$ group to a $=CH_2$ group decreases the bulk of the side chain and hence eliminates the "steric access" argument. If the serine hydroxyl plays an essential role in the enzyme action, such a modified chymotrypsin should be inert. If the serine hydroxyl acts as a scavenger, being acylated in competition with the water, its absence should not prevent water reaction, and an active enzyme would be expected.

Several alternative routes to a dehydroalanine derivative were considered. Phosphate compounds can lead to dehydroalanine, but it has been established that more than one product results.[13] A tosyl derivative seemed more desirable since Photaki[14] has shown that tosyl derivatives of serine can be converted to dehydroalanine and Fahrney and Gold[15] have shown that phenylmethanesulfonyl fluoride reacts with the active serine of chymotrypsin. Tosyl chloride was used in our experiments and was found to give a preferential tosylation of the active serine.

Preparation of tosyl-chymotrypsin: A solution of 500 mg of α-chymotrypsin in 50 ml of 0.2 N aqueous sodium chloride was kept at pH 7.0, while small portions of *p*-toluenesulfonyl-C^{14} chloride in dioxane were added at intervals. Time was allowed between individual additions for completion reaction to occur. The acid chloride additions were continued until the enzymatic activity had dropped to 5% of its original value as measured by the method of Schwert and Takenaka.[16]

The entire reaction mixture was subjected to gel filtration and the fractions corresponding to the peak in optical absorption (280 mμ) were combined and lyophilized to give 443 mg of fluffy white solid.

Radioassay of this material showed the presence of 0.99 toluenesulfonyl residues per molecule of chymotrypsin *p*-toluenesulfonate-C^{14}.

Kinetic study of the elimination of toluenesulfonic acid from tosyl-chymotrypsin: A solution of 40 mg of tosyl-chymotrypsin in 10.0 ml of water was cooled to 0° and treated with 10.0 ml of a cold 0.20 N sodium hydroxide solution with rapid mixing. The reaction mixture was kept at 0° and at various timed intervals 1 ml aliquots were added to 1.0 ml of cold 20% trichloroacetic acid solution to quench the reaction. From the measured radioactivities and the optical densities, it was possible to calculate the amount of radioactivity per mg of protein after the various timed intervals and thus the rate of elimination of the isotopically labeled toluenesulfonate group.

Preparation of anhydro-chymotrypsin: To an ice-cold solution of 300 mg of chymotrypsin *p*-toluenesulfonate-C^{14} in 25 ml of water was added rapidly, with swirling, 25 ml of ice-cold 0.20 N sodium hydroxide solution. After the mixture had stood in an ice bath for 4 hr, it was brought to pH 7.0 and subjected to gel filtration. The fractions corresponding to the peak in optical absorption (280 mμ) were combined and lyophilized to give 190 mg of fluffy white powder.

Radioassay of this material showed the presence of 0.22 toluenesulfonyl residues per molecule of anhydro-chymotrypsin. Essentially no loss and no gain in enzyme activity occurred during this detosylation procedure, i.e., the activity of the preparation was 5% after as well as before base treatment.

Base-treated chymotrypsin was prepared for control experiments by treatment of chymotrypsin exactly as described above for tosyl-chymotrypsin.

Results.—α-Chymotrypsin reacts readily with *p*-toluenesulfonyl chloride to form a tosylated derivative that has one toluenesulfonyl group per enzyme molecule. Several lines of reasoning led to the conclusion that this product is a sulfonic acid ester of the serine residue at the active site. First, it has been shown by Fahrney and Gold[15] that the analogous reaction of phenylmethanesulfonyl fluoride with chymotrypsin led to sulfonylation of the serine at the active site of the enzyme. Since both of these reactions led to inactivation of the enzyme, it appears that the same serine at the active site is tosylated in both cases. Secondly, the elimination experiments show that all but 10 per cent of the toluenesulfonyl group introduced into chymotrypsin via the sulfonyl chloride is easily removed by 0.1 N base at 0°.

Hydrolysis of sulfonamides (from lysine) and of sulfonic esters (from tyrosine) would not occur at an appreciable rate under these conditions, whereas an elimination reaction from the serine group[14] would have approximately this velocity. Finally, it should be noted that the group reacting with the sulfonyl chloride must have an unusual nucleophilicity, a property usually associated with the serine at the active site.

The tosyl-chymotrypsin has been shown by kinetic analysis to consist of three different substances. A plot of the extent of the elimination reaction versus time is shown in Figure 1, and it is clear that only 90 per cent of the toluenesulfonyl residue is eliminated. If the radioactivity remaining at 500 min is subtracted from earlier values, the corrected per cent losses shown in Figure 2 are obtained. The linearity of this log plot indicates that the majority of the protein molecules are present as a single species whose rate of elimination is indicated by the straight line. It is clear, however, that there is an initially rapid reacting material which is somewhat different from the bulk of the material present. The kinetic analysis indicates that about 12 per cent of the molecular species are in the especially reactive form, approximately 9 per cent in the inert form and 79 per cent in the form which eliminated at the velocity expected of a serine derivative. This analysis is further confirmed by a reaction with a tosyl-fluoride derivative which, from Fahrney and Gold's data, would be expected to be more selective for the serine residue and which was indeed found to react without producing any material which could not be eliminated by base.

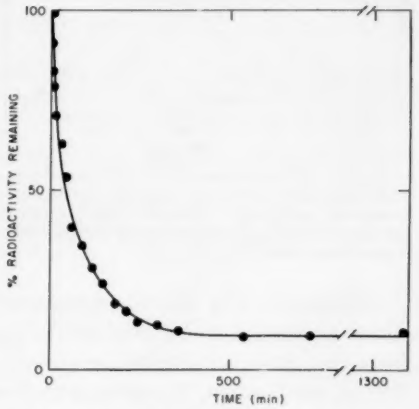


FIG. 1.—Elimination of the C^{14}-labeled tosyl group from tosyl-chymotrypsin on treatment with base.

The base treatment during the elimination reaction was shown not to result in denaturation of the enzyme. First, the small amount of enzyme activity (~5%) due to native chymotrypsin left in the tosyl-chymotrypsin preparation was virtually unchanged during the formation of anhydro-chymotrypsin. Second, similar base treatment of pure chymotrypsin resulted in a minor loss (less than 20%) of the enzymatic reactivity. Thus, the base treatment *per se* is not responsible for the presence of inactive enzyme. The inactivity of this modified chymotrypsin is therefore the result of the conversion of the serine residue to a dehydroalanine residue.

Despite the indirect evidence that the dehydroalanine had been prepared at the active site, it seemed desirable to demonstrate directly that such a residue was present. Therefore, several typical nucleophilic additions to an α,β-double bond were performed. The occurrence and extent of the addition reactions were determined by hydrolysis of the resulting protein and analysis for amino acid residues in the hydrolysate (cf. Table 1). The three addends used—sulfite ion, thioglycollic

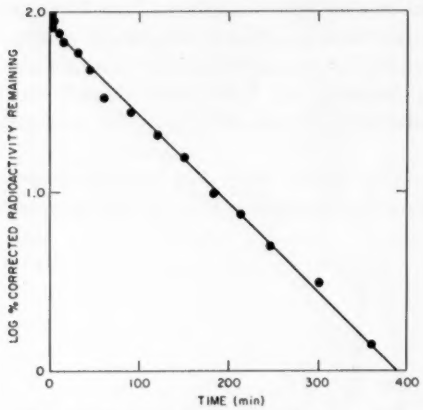


FIG. 2.—Elimination of C^{14}-labeled tosyl group from the active serine of tosyl-chymotrypsin on treatment with base. Data of Fig. 1 corrected for base-stable tosyl-chymotrypsin and plotted on logarithmic scale.

acid, and thiophenol—led to the formation of cysteic acid, carboxymethyl cysteine, and S-phenyl cysteine, respectively. Only small amounts of these products were formed when base-treated chymotrypsin was incubated with the adding reagents under the same conditions. These impurities may result from side reactions or perhaps an elimination reaction of the cysteine residues of the molecule. Thus, the finding that there were appreciably larger amounts of the adducts on treatment of anhydro-chymotrypsin as compared to base-treated chymotrypsin is corroborative evidence for the presence of a dehydroalanine residue in the anhydro-chymotrypsin.

Discussion.—The above experiments demonstrate that the conversion of the serine at the active site of chymotrypsin to dehydroalanine renders the molecule inactive. Since suitable controls establish that the treatments would not otherwise lead to inactivation, it is clear that it is the disappearance of the hydroxyl group which has led to the inactivation and hence that the hydroxyl group of serine plays a positive role in the action of chymotrypsin. The obvious role is that of a nucleophilic group at the active site. It might still be argued that the serine hydroxyl has a role in maintaining the tertiary structure of the active site, and this is a remote, but nevertheless finite, possibility. In any case, it is clear that the serine residue is not merely a scavenger which substitutes for the true acceptor, but is rather an essential group for the catalytic action of chymotrypsin.

Serine has also been implicated in the active center of a number of other esterases, in phosphoglucomutase, and in alkaline phosphatase. At times it has also been suggested that serine in these enzymes may play the part of a sink or scavenger rather than as a true catalytic group in the enzyme action. The evidence that serine plays a vital role in the catalysis of chymotrypsin therefore indicates that it probably plays a similar role in these other enzymes.

Finally, it is clear that the introduction of a double bond at the active site allows the possibility for the preparation of new types of derivatives of chymotrypsin. The adducts employed in the experiments described here were designed to demon-

TABLE 1

ADDITIONS TO THE DOUBLE BOND IN ANHYDRO-CHYMOTRYPSIN

Reagent to be added	Conditions	—Moles of Addition Product Produced— (Per Mole of Enzyme) Anhydro-chymotrypsin	Base-treated chymotrypsin
Na_2SO_3	20 hr, pH 9	0.56	0.21
Thioglycollic acid	24 hr, pH 8.2	0.95	0.55
Thiophenol	26 hr, pH 9.4	0.44	0.20

strate that such a double bond existed, but other adducts can be made whose properties may lead to a further understanding of the role of the serine residue in chymotrypsin. These studies will be published elsewhere.

Summary.—A modified chymotrypsin in which the serine residue at the active site has been transformed into a dehydroalanine residue has been prepared. This "anhydro-chymotrypsin" is inert. Since the serine modification in this case produces a smaller residue, the argument that serine modification prevents access to true catalytic residues is obviated. Therefore, it is concluded that serine cannot be a scavenger or alternate acceptor, but that the hydroxyl group of serine plays a direct positive role in the catalytic action of the enzyme.

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ON THE ASYMMETRY OF RNA SYNTHESIS IN VIVO

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The widely accepted thesis that RNA synthesis is required for the phenotypic expression of DNA-borne genetic information now has considerable experimental support.[1] Enzymes that catalyze RNA synthesis on a DNA template have also been isolated,[2–5] and these yield biosynthetic RNA capable of hybridizing to homologous DNA and of stimulating amino acid incorporation *in vitro*,[6, 7] although no

specific polypeptides have so far been synthesized. When the nature of the utilization of the DNA template for *in vitro* RNA synthesis was investigated, it was shown that both DNA strands are transcribed[5, 8] and yield self-complementary RNA.[9] Yet, our current knowledge of the amino acid code[10] makes virtually untenable the supposition that an RNA chain and its complement serve as messenger for the same polypeptide. It is also well established that ribosomal RNA is not self-complementary.[11] The experiments of Benzer and Champe[12] suggest that, whether or not both complementary segments of the DNA that constitutes the *r*II locus in phage T4 are transcribed, only one RNA species functions in its expression. The possibility that the *in vitro* and *in vivo* syntheses of RNA on a DNA template operate under different controls is therefore attractive to consider. In this communication we report experiments on the utilization of a viral genome for RNA synthesis in infected bacteria.

The experimental system is the following: shortly after infection of a fastidious strain of *B. megatherium* (Paris strain, also called *B. tiberias*) with a clear mutant of the lysogenic phage α, a new species of RNA is synthesized, which can be recognized by its ability to anneal with the DNA of phage α. The component polynucleotide strands of αDNA have sufficiently different nucleotide composition to permit their separation[13–15] and so afford the opportunity of testing the interaction of messenger RNA synthesized at various stages of the viral latent period with the separate strands of αDNA and with the RNA synthesized *in vitro* on a template of an isolated strand of αDNA. In this way we are able to compare the complementarity relationships of *in vivo* and *in vitro* synthesized αRNA.

Procedure.—1. *Preparation of phage α:* The mass preparation of phage α has been described previously.[13] Purification follows established lines: one cycle of low- and high-speed centrifugation, resuspension and high-speed centrifugation, treatment with DNase and RNase, a further cycle of differential centrifugation, and a final density gradient centrifugation in CsCl (ρ = 1.50), containing 0.005 *M* Mg^{++}.

2. *P^{32} labeling of infected and uninfected bacteria:* *B. megatherium* are grown to a density of 2.5–4 × 10^7/ml at 37°C in 250 ml dilute broth (0.5 gm NaCl, 1 gm meat extract, 1 gm peptone, 10 gm $MgSO_4$/liter, pH 7.2–7.4; doubling time 90 min). Phage α are added at a multiplicity of 30–50. In this medium, at 37°C, the latent period of the phage lasts 30–35 min; at some selected time during this interval, 3–7 mc carrier-free P^{32} are added. Five min after the addition of the label, the culture is poured onto ice, filtered through a thin pad of alumina on a nitrocellulose filter, washed with a buffer containing 7 gm Na_2HPO_4, 3 gm KH_2PO_4, 4 gm NaCl, and 0.2 gm $MgSO_4$/liter and finally with 10^{-2} *M* Tris,[33] pH 7.5, 10^{-4} *M* $MgCl_2$. The entire deposit, on its filter, is then frozen by placing it on a metal slab in a dry-ice–alcohol bath for 3–5 min. Uninfected bacteria are labeled and collected in the same way.

3. *Formation and detection of DNA-RNA hybrids:* Labeled RNA and denatured DNA (heated to 100°C for 5 min in 2 SSC or a lower-ionic-strength buffer, at a DNA concentration not exceeding 100 μg/ml) are mixed in the desired proportions and heated at 50°C for 4–12 hr in 2 SSC or 0.5 *M* KCl, 0.01 *M* Tris. The annealed solutions may then be stored frozen or analyzed immediately. Formation of DNA-RNA hybrids is detected by (*a*) retention of P^{32}-labeled RNA on nitrocellulose filters, or (*b*) DNA-dependent acquisition of ribonuclease resistance. The methods are as follows:

(*a*) *Nitrocellulose filter method of Nygaard and Hall:*[16] The sample to be analyzed is made up to a volume of 1 ml containing 150 μmoles NaCl (or KCl), 15 μmoles Na citrate, 10 μmoles Tris, pH 7.5, and 5 μg of RNase. It is incubated at 37°C for 15 min and added to 15 ml of 0.5 *M* KCl, 0.01 *M* Tris, passed through a nitrocellulose filter (Schleicher and Schuell, type A, coarse, 24-mm diameter, soaked in KCl-Tris for a minimum of 15 min before use), and washed with 60 ml KCl-Tris. The nitrocellulose filter, which retains RNA hybridized to denatured DNA, is dried and counted in an end-window Geiger-Mueller counter.

(*b*) *Ribonuclease resistance:* This method is also used for the detection of RNA-RNA hybrids,[9] for which the nitrocellulose filtration method is not suitable. The ribonuclease-digested sample as above is precipitated with 8% TCA using yeast RNA or denatured DNA carrier, collected on a Millipore filter, dried, and counted. For liquid scintillation counting, 70 μg heat-denatured salmon DNA serves as carrier in the TCA precipitation. The filter is rinsed with 2–3 ml 80% ethanol, dried, and counted in a scintillation medium of toluene, containing 4 gm/liter 2,5-diphenyloxazole and 0.1 gm/liter (4-methyl, 5-phenyloxazolyl) benzene.

4. *The purification of P^{32} pulse-labeled α-specific RNA* follows in these steps:

(*a*) Frozen bacteria (*Procedure*, 2) are ground with alumina in a mortar, suspended in a buffer containing 0.1 *M* acetate, pH 5.2, 0.5% sodium dodecyl sulfate, and 500 μg/ml bentonite, and extracted three times with hot phenol.[17] After ether extraction, the aqueous phase is neutralized and heated 5 min at 100°C. KCl is added to 0.5 *M*, and the solution filtered through nitrocellulose membranes.[16] The filtrate is then passed through a column of medium-grade Sephadex G75, pre-equilibrated with 2 SSC. The macromolecular (front) component of the eluate contains RNA uncontaminated by labeled or unlabeled DNA as judged by the following criteria: after self-annealing, CsCl density gradient centrifugation shows no band of P^{32} in the region of DNA buoyant density; P^{32}-labeled material is 95% converted to TCA solubility by digestion with pancreatic ribonuclease.

(*b*) The total RNA is now annealed with denatured αDNA, at 10–40 μg/ml, for 6–12 hr in 2 SSC at 50°C.

(*c*) DNA-RNA hybrid and denatured DNA are separated from the remainder of the RNA.

(*d*) α-specific RNA is separated from αDNA.

Steps (*c*) and (*d*) have been executed in one of two ways:

(1) The annealed mixture from step (*b*) is applied to a column of nitrocellulose in 0.5 *M* KCl.[18] Under these conditions unannealed RNA passes through the column while DNA and DNA-RNA hybrid are retained. The DNA and hybrid are then eluted from the column with 0.005 *M* Tris, first at 25°C and then at 37°C. The pooled eluates are concentrated, dialyzed, denatured at 100°C, and the RNA is separated from DNA by passing repeatedly through a nitrocellulose filter [*Procedure*, 3(*a*)] in 0.5 *M* KCl, 0.01 *M* Tris. In this way, the DNA is retained on the filter; the RNA passes through and is collected.

(2) The annealed mixture from step (*b*) in 0.5 *M* KCl, 0.01 *M* Tris, is passed through nitrocellulose filters, which retain DNA and DNA-RNA hybrid. The quantity of annealing mixture applied to each filter is such as to deposit less than 100 μg DNA. The filters are washed with 0.5 *M* KCl, 0.01 *M* Tris, pH 7.5, and are then extracted with 0.005 *M* Tris, pH 7.5, at 60–100°C. The extracted DNA and RNA are then completely dissociated by denaturation and separated by further nitrocellulose filtration.

At the present time, both of these simple procedures which provide a satisfactory purification (cf. Figs. 2*b* and 3) suffer from one disadvantage: RNA is annealed with a relatively high concentration of DNA in order to extract as high a fraction of the α-specific component as possible. Under these conditions, the filters are loaded to their DNA capacity, and it is difficult to achieve a complete re-extraction of the labeled RNA. Elution from the column suffers a similar drawback.

5. *Purification of the light strand of αDNA (α_LDNA):* Kieselguhr coated with methylated serum albumin is prepared according to the procedure of Mandell and Hershey,[19] formed into a column 1.2 × 3 cm, as described by Sueoka and Cheng,[20] and washed with 0.2 *M* NaCl, 0.25 *M* phosphate pH 7.9. αDNA is denatured in SSC at a concentration of 20 μg/ml, applied to the column, and washed with 0.2 *M* NaCl, 0.25 *M* phosphate. Stepwise elution follows, starting with 0.2 *M* NaCl, 0.25 *M* Na phosphate pH 7.9, and increasing the NaCl concentration by steps of 0.05 *M*.[21] α_LDNA is the first material to be eluted from the column, being released in 0.30–0.35 *M* NaCl. However, in our hands, neither the yield nor the purity of the α_LDNA is a consistent feature of the fractionation. Accordingly, the freedom from contamination of α_LDNA by its complement is verified by self-annealing a portion at 60°C for 6 hr in 2 SSC at a DNA concentration of 15–25 μg/ml and centrifuging in 7.7 *M* CsCl against a density marker of *M. lysodeikticus* DNA. We use only those preparations of α_LDNA which, after annealing, have the same buoyant density as the light component of denatured total αDNA (ρ = 1.716 relative to ρ = 1.731 assigned to *M. lysodeikticus*) and which contain no material having the buoyant densities of α_H (ρ = 1.724) or native α (ρ = 1.705) DNA. Under the same annealing conditions, denatured (total) αDNA

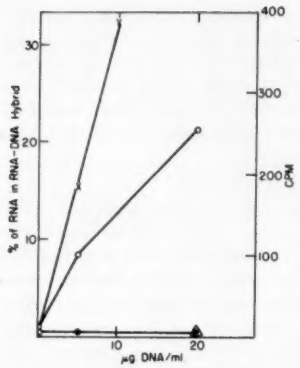


FIG. 1.—The ability of P^{32}-labeled αCRNA to form DNA-RNA hybrids with its homologous αDNA (○), the light strand of αDNA (X), and with heterologous *B. megatherium* (●) and T7 (△) DNA. The conditions of hybrid formation are: denatured αCRNA 10 μg/ml, all DNA except α_L also denatured, solvent 2 SSC, annealing 50°C, 6 hr. RNA-DNA hybrid detection on nitrocellulose filters.

reforms a band of density 1.706. The ability of α_LDNA to form RNA-DNA hybrids is demonstrated in Figure 1.

6. *Synthesis of αCRNA:* RNA complementary to total αDNA (αCRNA) labeled with P^{32} was synthesized and isolated as described elsewhere,[8] using CRP^{32} PP or URP^{32} PP as the labeled nucleoside triphosphates.

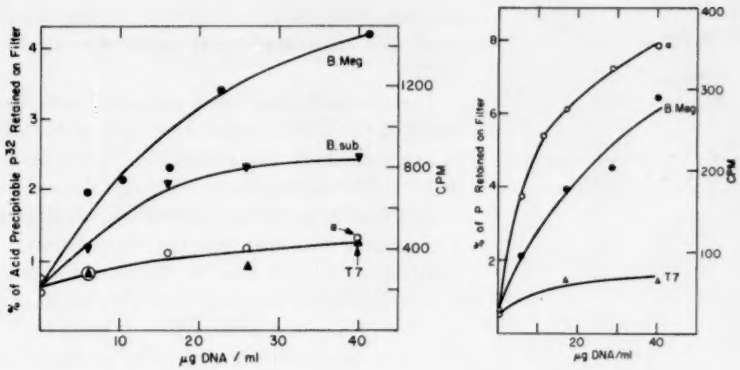
7. *DNA:* α and T7 DNA were prepared from CsCl gradient purified phage by the method of Mandell and Hershey.[19] *B. megatherium* DNA was prepared by chloroform-isoamyl alcohol extraction of bacteria lysed in 0.37 *M* sucrose, 10^{-3} *M* $MgCl_2$, Tris-phosphate pH 8.3, by lysozyme at 5°C. In other respects the isolation followed the procedure of Marmur,[22] except that his final chloroform extraction and precipitation were replaced by at least three phenol extractions (to ensure complete removal of ribonuclease) and dialysis.

8. *Synthesis of RNA complementary to the light strand of αDNA ($\alpha_H RNA$):* For this purpose, α_L DNA is used to prime RNA polymerase. In one such synthesis the reaction mixture contained 7 μg α_LDNA; 300 μmoles Tris, pH 7.5; 2.4 μmoles each of ATP, UTP, GTP, and spermidine; 0.3 μmoles CTP-H^3 at a specific activity of 160 c/mole; 30 μmoles $MgCl_2$; and 180 μg RNA polymerase isolated from *M. lysodeikticus*[23] in a total volume of 3 ml. Incubation was at 30°C for 45 min and yielded 9 μg of TCA precipitable polyribonucleotide. The reaction mixture was extracted twice with phenol, then with ether, and dialyzed, first against 1 *M* NaCl, 0.01 *M* Tris, pH 7.5, and then against 0.01 *M* Tris. The mixture of RNA and DNA was denatured and centrifuged in a Cs_2SO_4 density gradient to separate the RNA from DNA. RNA was collected from the denser region of the gradient, dialyzed, and stored at −20°C.

9. *Determination of the distribution of P^{32}* in the nucleoside-2′ (3′) phosphates of alkaline digests of RNA was performed as described elsewhere.[2]

Results and Discussion.—The method of RNA-DNA hybrid formation clearly identifies a new species of RNA in cells of *B. megatherium* infected with phage α. Figure 2*a* shows the pattern of RNA–DNA interactions for a 5-min P^{32}-labeled RNA preparation isolated from *uninfected B. megatherium* There is interaction with the DNA from *B. megatherium* and a high degree of cross reaction with *B. subtilis* DNA, but little or no hybrid formation with T7 or αDNA. For the purpose of these experiments, we define background in terms of the interaction of RNA with T7 bacteriophage DNA. This background, which is only slightly above that provided by the RNA in the absence of DNA, is probably due to physical entrapment of RNA by the deposited DNA, although the possibility of accidental homology of short ribonucleotide sequences with heterologous DNA is not excluded. By contrast, Figure 2*b* shows the pattern of hybridization for the total RNA pulse-labeled from the 20th to the 25th min *after infection of B. megatherium* with phage α. That portion of the RNA which hybridizes with αDNA is further purified as described in *Procedure* 3, and its properties will be described below. At 10–15 and 15–20 min (data not shown), a similar pattern of interactions is observed. In all cases there is RNA–DNA hybrid formation with total viral DNA, but a certain fraction of the RNA anneals with the DNA of the host.

On the other hand, αCRNA shows no cross reaction with host DNA (Fig. 1).



(*a*) (*b*)

FIG. 2.—The ability of pulse-labeled RNA from uninfected and infected *B. megatherium* to form DNA-RNA hybrids with α, *B. megatherium*, *B. subtilis*, and T7 DNA. (*a*) Uninfected log phase bacteria P^{32} labeled for 5 min. The conditions of hybrid formation are: RNA (*Procedure*, 4, step *a*) 6 μg/ml; denatured DNA; solvent 2 SSC; annealing 50°C, 6 hr. RNA-DNA hybrids detected on nitrocellulose filters after RNase digestion. (*b*) Pulse label 20–25 min after infection. RNA 1.4 μg/ml (*Procedure*, 4, step *a*); anneal 50°C, 6 hr in 2 SSC. Hybrids detected on nitrocellulose filters after RNase digestion.

The interaction with *B. megatherium* DNA cannot therefore be due to accidental homology. It represents either continuing host RNA synthesis in infected cells or else RNA synthesis in abortively infected cells.

The ability of purified αRNA (*Procedure*,§ 4, step *d*, method 1) to anneal with α, α_L, and *B. megatherium* DNA is shown in Table 1 and Figure 3 (method 2).

TABLE 1

HYBRID FORMATION BY PURIFIED αRNA PULSE-LABELED 10–15 MINUTES AFTER INFECTION

Anneal 6 hr at 50°C with	Hybrid assay % of P^{32} retained on filter
10 μg/ml αDNA	21
5 μg/ml α_LDNA	3
10 μg/ml *B. megatherium* DNA	3
No DNA	2

The lack of interaction of the purified αRNA with host DNA is a measure of the success of this particular purification method. The inability of the αRNA to anneal with α_LDNA is also strikingly illustrated. The effect is even more pronounced when one bears in mind that α_LDNA anneals even better to αCRNA than does αDNA (Fig. 1). We assume that this is due to competition in the latter case between DNA-RNA and DNA-DNA complex formation. If the same situation exists in the αRNA annealing experiments of Table 1, then the relative frequency of polynucleotide sequences on α_LDNA which are complementary to αRNA cannot be greater than 1:20. In the experiment shown in Figure 3 we were unable to detect any α_L complementarity in αRNA. On the other hand, αRNA does interact with the α_HRNA which has been synthesized enzymatically on a template of α_LDNA (Fig. 3). This eliminates the possibility that we might have prepared, as our α_LDNA, a fragment of the corresponding entire polynucleo-

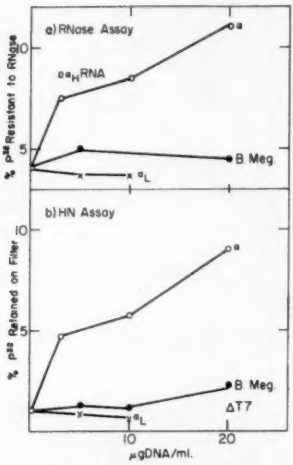


FIG. 3.—Interaction of purified αRNA from pulse (15–20 min after infection) with α, α_L, and *B. megatherium* DNA and α_HRNA. Detection of hybrids (*a*) by RNase resistance and (*b*) on nitrocellulose filters.

tide strand of the virus and that neither that fragment nor its complement codes for 10–15-min αRNA.

These results establish the non-self-complementarity of the RNA purified from cells pulse-labeled at approximately the midpoint of viral development. The distribution of P^{32} in 2′(3′) nucleotides in an alkaline digest of purified αRNA gives a result entirely in accord with this supposition. The distribution is very close to the base composition of α_LDNA and is complementary to the base composition of α_HDNA as reported by Marmur (Table 2). The agreement is especially gratifying since we do not have data on the saturation of the annealing of purified αRNA. On the other hand, the corresponding unfractionated RNA from the infected bacteria has a distinctly different distribution of P^{32} among the 2′(3′) nucleotides.

These experiments permit the following statements: the phage-specific RNA purified after a pulse-label administered during viral development fails to interact with one strand of the viral DNA but does interact with its complement. The isotope-"base" composition of this isolated RNA is like that of one of the strands of the viral DNA, and the isolated RNA interacts with the synthesized complement of that strand. The *in vivo* synthesized messenger RNA is obviously not self-complementary. Yet the messenger complement, or "anti-messenger," can be synthesized *in vitro* either on a template of native DNA or on the appropriate single DNA polynucleotide strand. Clearly the *in vitro* and *in vivo* syntheses operate under different control. The alternative possibilities regarding the nature of this difference are that (*a*) the turnover rate of one RNA strand is completely different from the other, so that only one can be detected within our pulse-label periods; the two extreme possibilities are nonsynthesis or very slow synthesis of the α_H-RNA-like strand on the one hand, or its very rapid breakdown on the other; and (*b*) both chains are synthesized at a comparable rate, and one is so bound as not to be isolated by our particular method. Within the scope of the current experiments, these alternatives cannot be clearly distinguished. However, the hot-phenol RNA extraction procedure is known to yield both particulate (ribosomal and ribosome-bound) as well as supernatant RNA, so that alternative (*b*) appears intrinsically the less likely.

The work of Hall, Green, Kano-Sueoka, and Spiegelman establishes that the timing of the synthesis of various viral proteins in T4-infected *E. coli* has its counterpart in the synthesis of demonstrably different RNA molecules at different times.[24, 25] It is likely that during the development of phage α also, only a small number of genes, or gene clusters, are transcribed within the span of a brief labeling period. For two such labeling periods, we show complementarity of RNA with only one strand of the DNA. However, we have yet to provide a comparison of pulse-labeling periods distributed over the entire developmental cycle of phage α.

TABLE 2

COMPARISON OF THE BASE COMPOSITION OF αDNA WITH THE DISTRIBUTION OF P^{32} IN ALKALI DIGESTS OF PULSE-LABELED RNA

	Mole per cent				
	G	A	C	U(T)	Ref.
α_LDNA	24.1	30.1	21.3	24.5	29
α_HDNA	19.9	24.0	24.2	32.1	29
Total RNA of infected cells*	23.5	28.5	20.0	28.0	This work
αRNA*	25.5	31	20.5	23	This work

* P^{32} distribution; RNA pulse-labeled 10–15 min after infection.

At a recent symposium we reported briefly on the initial results of this investigation.[26] At the same time, parallel work on other systems was also discussed. Experiments on the properties of virus-specific messenger synthesized in bacteria infected with the virulent viruses T4,[27, 28] SP8,[29] and ϕX174[30] and on uninfected *E coli*[31] lead to conclusions which are entirely similar to ours. A striking experiment which can be interpreted along identical lines has been performed by Guild and Robison,[32] who have partly purified the individual strands of *D. pneumoniae* DNA corresponding to the complementary pair of a single genetic marker. When these two versions of the genetic material are taken up by recipient bacteria, the time of their first phenotypic expression differs. These experiments provide clear evidence, as do ours, for a basic asymmetry of the *in vivo* synthesis of RNA on a DNA template.

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‡ Department of Biochemistry and Argonne Cancer Research Hospital, the University of Chicago. The ACRH is operated by the University of Chicago for the U.S. Atomic Energy Commission.

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A STABILITY THEOREM FOR FUNCTIONAL-DIFFERENTIAL EQUATIONS

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The term functional-differential equation as used in this paper is a natural generalization of the concept of a differential equation with delayed arguments. As pointed out by Krasovskii,[1] the natural setting for a discussion of such equations seems to be in the space C of continuous functions over a finite interval. Krasovskii has shown that the stability theorems of Lyapunov and their converses can be extended if the Lyapunov functions are defined on the space C. These theorems yield necessary and sufficient conditions for stability and play an important role in the investigation of the theoretical implications of stability. Unfortunately, the application of these results to determine the stability properties of a particular equation is not practical, and it is important to determine conditions by means of Lyapunov functionals which are only sufficient for stability (see ref. 1 for a detailed bibliography).

For ordinary differential equations, LaSalle[2] has shown that the limiting sets of bounded trajectories are contained in the largest invariant set contained in the set where the derivative of a Lyapunov function V vanishes, provided only that V is

bounded below and the set where V is less than a constant is bounded. It is the purpose of the present note to extend this result to functional-differential equations.

Let R^n be an n-dimensional vector space with norm $|\cdot|$, and let $C = C([-r,0], R^n)$ be the space of continuous functions taking the interval $[-r,0]$ into R^n. For any φ in C, define the norm of φ, $\|\varphi\|$, to be $\sup_{-r\leq\theta\leq 0}|\varphi(\theta)|$. If x is any continuous function defined on the interval $[-r,A)$, $A > 0$, then x_t will denote an element of C for each t in $[0, A)$ defined by $x_t(\theta) = x(t+\theta)$, $-r \leqq \theta \leqq 0$; that is, x_t is that "segment" of the graph of $x(t)$ which is obtained by letting t vary from t-r to t. If the operation "$\cdot$" denotes right hand derivation and f is any function mapping an open subset S of C into R^n, then a functional-differential equation is a relation of the form

$$\dot{x}(t) = f(x_t),\ t \geqq 0. \tag{1}$$

A *solution of (1) with initial function φ in C at 0* is a function $x(\varphi)$ defined and continuous on an interval $[-r,A)$, $A > 0$ such that $x_t(\varphi)$ is in S for t in $[-r,A)$, $x_0(\varphi) = \varphi$ and $x(\varphi)(t)$ satisfies (1) for $0 \leqq t < A$. If $f(\varphi)$ is locally Lipschitzian in S, then one can show that for any φ in S, there is one and only one solution $x(\varphi)$ of (1) with initial function φ at 0 and this function depends continuously upon φ. Moreover, $x(\varphi)(t)$ has a continuous derivative for $t > 0$ and can be continued in t as long as $x_t(\varphi)$ remains in S. In the following, we always suppose that $S = \{\varphi \text{ in } C : \|\varphi\| < H\}$ where H is some positive number (maybe ∞), $f(0) = 0$ and f is locally Lipschitzian in S. Equation (1) with f satisfying these hypotheses will be briefly referred to as system (1). Therefore, all stability criteria will be relative to the set S. When $H = \infty$, then $S = C$ and we have global stability.

It is quite natural to consider system (1) as defining motions or paths in C. In fact, we can define a *motion through φ* as the set of functions in C given by $\bigcup_{0\leq t<A} x_t(\varphi)$ where the interval $[-r,A)$ is the region of definition of $x(\varphi)$. An element ψ of C is in $\Omega(\varphi)$, the *ω-limit set of φ*, if $x(\varphi)$ is defined on $[-r,\infty)$ and there is a sequence of nonnegative real numbers t_n, $t_n \to \infty$ as $n \to \infty$ such that $\|x_{t_n}(\varphi) - \psi\| \to 0$ as $n \to \infty$. A set M in C is called an invariant set if for any φ in M, the solution $x(\varphi)$ is defined on $(-\infty,\infty)$ and $x_t(\varphi)$ is in M for t in $(-\infty,\infty)$. Notice that solutions on an invariant set must be defined on $(-\infty,\infty)$, a property which is not enjoyed by all solutions of even the simplest form of system (1).

For any $H_1 < H$, there is a constant L such that $|f(\varphi)| \leqq L$ for all φ with $\|\varphi\| \leqq H_1$. From this, one easily obtains the following result:

LEMMA 1. *If $x(\varphi)$ is a solution of system (1) with initial function φ at 0, defined on $[-r,\infty)$ and $\|x_t(\varphi)\| \leqq H_1 < H$ for all t in $[0,\infty)$, then the family of functions $\{x_t(\varphi), t \geqq 0\}$ belongs to a compact subset of C; that is, the motion through φ belongs to a compact subset of C.*

From this lemma and the standard type of argument used in the theory of dynamical systems, we obtain

LEMMA 2. *If φ in S is such that the solution $x(\varphi)$ of system (1) with initial function φ at 0 is defined on $[-r,\infty)$ and $\|x_t(\varphi)\| \leqq H_1 < H$ for t in $[0,\infty)$, then $\Omega(\varphi)$ is a nonempty, compact, invariant set and dist$(x_t(\varphi), \Omega(\varphi)) \to 0$ as $t \to \infty$.*

For the statement of the main result of this note, it is convenient to introduce some additional notation. If V is a continuous scalar function defined on S, then

the derivative of V along the solutions of (1) will be designated by $\dot{V}_{(1)}$ and is defined as

$$\dot{V}_{(1)}(x_t(\varphi)) = \overline{\lim_{h \to 0^+}} \frac{1}{h} [V(x_{t+h}(\varphi)) - V(x_t(\varphi))]. \tag{2}$$

For any $s \leqq r$ and φ in C, define

$$\|\varphi\|_{[-s,0]} = \sup_{-s \leq \theta \leq 0} |\varphi(\theta)|. \tag{3}$$

If $s < r$, (3) is a pseudo-norm for C and if $s = r$, (3) yields the norm we have been using for C.

THEOREM 1. *Let V be a continuous scalar function on S. If U_l designates the region where $V(\varphi) < l$, suppose there exist nonnegative constants K,a such that $\|\varphi\|_{[-a,0]} \leqq K$, $V(\varphi) \geqq 0$, and $\dot{V}_{(1)}(\varphi) \leqq 0$ for all φ in U_l. If R is the set of all points in U_l where $\dot{V}_{(1)}(\varphi) = 0$ and M is the largest invariant set in R, then every solution of (1) with initial value in U_l approaches M as $t \to \infty$.*

This theorem together with Theorem 3 below generalize results of LaSalle[3] for ordinary differential equations and the proofs are natural extensions of the ones given by LaSalle.

Proof: The conditions of V imply that $V(x_t(\varphi))$ is a nonincreasing function of t and $V(x_t(\varphi))$ is bounded below within U_l. Hence, φ in U_l implies $x_t(\varphi)$ in U_l and $\|x_t(\varphi)\|_{[-a,0]} \leqq K$ for all $t \geqq 0$ which implies $\|x_t(\varphi)\|_{[-r,0]} \leqq K$ for all $t \geqq 0$; that is, $x_t(\varphi)$ is bounded and Lemma 2 implies $\Omega(\varphi)$ is an invariant set. But $V(x_t(\varphi))$ has a limit $l_0 < l$ as $t \to \infty$ and $V = l_0$ on $\Omega(\varphi)$. Hence, $\Omega(\varphi)$ is in U_l and $\dot{V}_{(1)} = 0$ on $\Omega(\varphi)$. Consequently, $\Omega(\varphi)$ invariant implies $\Omega(\varphi)$ is in M and Lemma 2 implies $x_t(\varphi) \to M$ as $t \to \infty$, completing the proof of the theorem.

COROLLARY 1. *If the conditions of Theorem 1 are satisfied and $\dot{V}_{(1)}(\varphi) < 0$ for all $\varphi \neq 0$ in U_l, then every solution of (1) with initial value in U_l approaches 0 as $t \to \infty$.*

Corollary 1 does not seem to imply anything about the stability of the solution $x = 0$, but only asserts the asymptotic approach to zero. The following result due to Krasovskii[4] concerns asymptotic stability.

THEOREM 2. *Suppose there exist functions $v(s)$, $w(s)$ which are continuous and increasing for $0 \leqq s < H$ with $v(0) = w(0) = 0$. In addition, assume there is a constant b, $0 \leqq b \leqq r$, and a continuous scalar function $V(\varphi)$ defined on S such that*

$$v(\|\varphi\|_{[-b,0]}) \leqq V(\varphi) \leqq w(\|\varphi\|_{[-r,0]}) \tag{4a}$$

$$\dot{V}_{(1)}(\varphi) < 0, \quad \varphi \neq 0, \tag{4b}$$

for all φ in S. Under these conditions, the solution $x = 0$ of (1) is asymptotically stable and every solution of (1) approaches 0 as $t \to \infty$, provided the initial value φ satisfies $V(\varphi) < l_0$, $l_0 = \lim_{r \to H} v(r)$.

Proof: Since v is increasing the set U_l of φ for which $V(\varphi) < l$ satisfies the conditions of Theorem 1 if $l < l_0$ and, thus, by Corollary 1, every solution in U_l approaches zero as $t \to \infty$. To prove $x = 0$ is stable, choose $\epsilon > 0$ arbitrary and $\delta > 0$ so small that $w(\delta) < v(\epsilon)$. For any φ with $\|\varphi\| < \delta$ it follows from the hypothesis on $\dot{V}_{(1)}$ that $\|x_t(\varphi)\|_{[-b,0]} < \epsilon$ for all $t \geqq 0$ which implies $\|x_t(\varphi)\|_{[-r,0]} < \epsilon$ for all $t \geqq 0$ completing the proof of the theorem.

THEOREM 3. *Let $S = C$ and V be a continuous scalar function on C. If $V(\varphi) \geqq 0$,*

$\dot{V}_{(1)}(\varphi) \leqq 0$ for all φ in C and R is the set of φ in C for which $\dot{V}_{(1)} = 0$ and M is the largest invariant set in R, then all bounded solutions of (1) approach M as $t \to \infty$.

If, in addition, there exist a constant $c, 0 \leqq c \leqq r$, and a function $u(s)$, nonnegative and continuous for $0 \leqq s < \infty$, $u(s) \to \infty$ as $s \to \infty$, such that

$$u(\|\varphi\|_{[-c,0]}) \leqq V(\varphi) \tag{5}$$

for all φ in C, then all solutions of (1) are bounded for all $t \geqq 0$.

Proof: The first part of the theorem proceeds essentially as in Theorem 1. The boundedness property proceeds as follows. For any φ_0 in C, there is a constant m such that $V(\varphi) > V(\varphi_0)$ for $\|\varphi\|_{[-c,0]} \geqq m$. Since $V(x_t(\varphi_0))$ is a nonincreasing function of t, it follows that $\|x_t(\varphi_0)\|_{[-c,0]} < m$ for all $t \geqq 0$ which implies $\|x_t(\varphi_0)\|_{[-r,0]} < m$ for all $t \geqq 0$ and the theorem is proved.

COROLLARY 2. *If all of the conditions of Theorem 3 are satisfied and $\dot{V}_{(1)} < 0$ for $\varphi \neq 0$, then all solutions of (1) approach zero as $t \to \infty$. If, in addition, (4a) is satisfied, the origin is globally asymptotically stable.*

We now give some applications of these results.

Example 1: Suppose $n = 1$ and

$$f(\varphi) = -\frac{1}{r}\int_{-r}^{0} g(\varphi(\theta))(r + \theta)d\theta$$

where $g(x)$ is a real function defined for all real x, locally Lipschitzian in x and

$$xg(x) > 0, \; G(x) = \int_0^x g(\xi)d\xi \to \infty \text{ as } |x| \to \infty. \tag{6}$$

System (1) is then given by

$$\dot{x}(t) = -\frac{1}{r}\int_{-r}^{0} (r + \theta)g(x(t + \theta))d\theta, \; t \geqq 0. \tag{7}$$

We wish to prove

THEOREM 4 (Levin and Nohel[5]). *Each solution of (7) approaches a solution, which is necessarily periodic, of the second order ordinary differential equation*

$$\ddot{x}(t) + g(x(t)) = 0. \tag{8}$$

Furthermore, if $x(t,\alpha)$ is a solution of (8) with $x(0,\alpha) = \alpha$, $\dot{x}(0,\alpha) = 0$, with period $p(\alpha)$, then it satisfies (7) if and only if $mp(\alpha) = r$ for some integer m.

Proof: To prove the first part of the theorem, we make use of the scalar functional $V(\varphi)$ introduced by Levin and Nohel, namely,

$$V(\varphi) = G(\varphi(0)) + \frac{1}{2r}\int_{-r}^{0}\left[\int_{\tau}^{0} g(\varphi(\theta))d\theta\right]^2 d\tau,$$

which has the property that

$$\dot{V}_{(7)}(\varphi) = -\frac{1}{2r}\left[\int_{-r}^{0} g(\varphi(\theta))d\theta\right]^2 \leqq 0.$$

The conditions of Theorem 3 are satisfied and the set R is the set of all φ such that $\int_{-r}^{0} g(\varphi(\theta))d\theta = 0$ and the set M consists of points $x_t(\varphi)$ where $x(\varphi)$ is a solution of

(7) such that

$$\int_{-r}^{0} g(x(\varphi)(t+\theta))d\theta = 0. \tag{9}$$

But if x is a solution of (7), then

$$\ddot{x}(t) + g(x(t)) = \frac{1}{r}\int_{-r}^{0} g(x(t+\theta))d\theta$$

and, thus, M is generated by those periodic solutions of (8) which satisfy (9). The fact that these solutions of (8) satisfy the properties mentioned in the theorem follows by a direct computation on (8).

Example 2:[6]

$$\begin{aligned}\dot{x}(t) &= y(t)\\ \dot{y}(t) &= -f(y(t)) - g(x(t)) + \int_{-r}^{0} h(x(t+\theta))y(t+\theta)d\theta\end{aligned} \tag{10}$$

where

$$f(x)/x > b > 0,\ g(x)/x > a > 0,\ |h(x)| < L,\ r < b/L,$$

for all x. If $h(x) = df(x)/dx$, system (10) is related to a second order differential-difference equation. For any scalar functions φ, ψ defined and continuous on $[-r,0]$, define

$$V(\varphi,\psi) = 2G(\varphi(0)) + \psi^2(0) + \nu\int_{-r}^{0}\left(\int_{\tau}^{0}\psi^2(\theta)d\theta\right)d\tau \tag{11}$$

where $G(x) = \int_0^x g(s)ds$ and ν is a positive constant to be determined.

A direct computation shows that

$$\dot{V}_{(10)}(\varphi,\psi) \leqq -\int_{-r}^{0}\left[\left(\frac{2b}{r} - \nu\right)\psi^2(0) - 2L|\psi(0)\psi(\theta)| + \nu\psi^2(\theta)\right]d\theta. \tag{12}$$

If $r < b/L$, then one can always find a positive ν for which the integrand is positive definite in $\psi(0)$, $\psi(\theta)$ which implies $\dot{V}_{(10)}(\varphi,\psi) = 0$ if and only if $\psi = 0$. But, the only invariant set of (10) which lies in the set of (φ,ψ) for which $\psi = 0$ is (0,0). Consequently, the above theorems may be applied to obtain the result that the solution $x = 0$, $y = 0$ of (10) is asymptotically stable and if, in addition $G(x) \to \infty$ as $|x| \to \infty$, this solution is globally asymptotically stable.

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*A NEW TYPE OF NONASSOCIATIVE ALGEBRAS**

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A problem of interest in nonassociative algebras is the study of generalized Cayley algebras and exceptional simple Jordan algebras which are closely related to the exceptional simple Lie algebras. Thus, the derivations of a generalized Cayley algebra form an exceptional Lie algebra of type G, the derivations of an exceptional simple Jordan algebra form an exceptional Lie algebra of type F, and the derivations and right multiplications of elements of trace zero of an exceptional simple Jordan algebra generate an exceptional Lie algebra of type E_6.[1] Furthermore, all of these representations involve the representations of lowest possible dimension for the respective Lie algebras. The lowest possible dimension for a representation space for the exceptional Lie algebra $\mathfrak{E}_7$ is 56. Starting from the class of exceptional simple Jordan algebras, we will define a new class of simple nonassociative algebras of dimension 56 over their centers and possessing nondegenerate trace forms, such that the derivations and left multiplications of elements of trace zero generate Lie algebras of type E_7. Some basic properties of these algebras will be given without proof (most of the proofs consist of direct computations).

Definition. *Let $\mathfrak{J}$ be an exceptional simple Jordan algebra over a ground field $\mathfrak{F}$ of characteristic not two or three. We define the algebra $\mathfrak{N}$ to consist of all quadruples (α, β, a, b) with α, β in $\mathfrak{F}$ and a, b in $\mathfrak{J}$. Set $f_1 = (1, 0, 0, 0)$, $f_2 = (0, 1, 0, 0)$, $a_{12} = (0, 0, a, 0)$, $b_{21} = (0, 0, 0, b)$. Furthermore, let $a \times b$ denote the cross product on $\mathfrak{J}$ and $(a, b) = tr\,(ab)$ the trace form on $\mathfrak{J}$.[2] Then the product in the algebra $\mathfrak{N}$ is defined as follows:* $f_1^2 = f_1$, $f_1f_2 = f_2f_1 = 0$, $f_2^2 = f_2$, $f_1a_{12} = \mu a_{12}$, $f_2a_{12} = (1 - \mu)a_{12}$, $f_1b_{21} = (1 - \mu)b_{21}$, $f_2b_{21} = \mu b_{21}$, $a_{12}f_1 = (1 - \nu)a_{12}$, $a_{12}f_2 = \nu a_{12}$, $b_{21}f_1 = \nu b_{21}$, $b_{21}f_2 = (1 - \nu)b_{21}$, $a_{12}c_{12} = \omega_1(a \times c)_{21}$, $b_{21}d_{21} = \omega_2(b \times d)_{12}$, $a_{12}b_{21} = \delta_1(a, b)f_1$, $b_{21}a_{12} = \delta_2(a, b)f_2$. *The parameters $\omega_1, \omega_2, \delta_1, \delta_2$ in $\mathfrak{F}$ are assumed to be nonzero, while the possibility that μ or ν in $\mathfrak{F}$ is zero is allowed.*

The algebra $\mathfrak{N}$ is central simple and is *not* associative, flexible, or power-associative. The element $f = f_1 + f_2$ is a unity element, and the left, middle, and right nuclei of $\mathfrak{N}$ all consist of scalar multiples of f only. A *trace* may be defined on $\mathfrak{N}$ as follows. If $x = \alpha f_1 + \beta f_2 + a_{12} + b_{21}$, we set $tr\,(x) = \alpha + \beta$. Then $(x, y) = {}^1/_2\, tr(xy + yx)$ is a nondegenerate symmetric bilinear *trace form* on $\mathfrak{N}$. (If $\delta_1 = \delta_2$, we may define (x, y) as simply $tr(xy)$.) We have $(xy, z) = (x, yz)$ for all x, y, z in $\mathfrak{N}$ if and only if $\delta_1 = \delta_2$ and $\mu = \nu = 1$. However, this particular special case is not of too much interest to us, because the derivations and left multiplications of elements of trace zero *do not* generate a Lie algebra of type E_7. On the other hand, without any restrictions on the parameters $\omega_1, \omega_2, \delta_1, \delta_2, \mu, \nu$ other than that the first four be nonzero, the derivation algebra of $\mathfrak{N}$ is a Lie algebra of type E_6, as is seen by the following theorem.

Theorem 1. *If $\omega_1, \omega_2, \delta_1, \delta_2$ are not zero and the characteristic of the ground field is not two or three, then the derivations D of the algebra $\mathfrak{N}$ are given by D:* $f_1 \to 0, f_2 \to 0$, $a_{12} \to (aD_{12})_{12}$, $b_{21} \to (-bD_{12}{}^*)_{21}$, *where D_{12} is a linear transformation on $\mathfrak{J}$ contained in the Lie algebra generated by the derivations of $\mathfrak{J}$ and the multiplications of elements*

of trace zero in $\mathfrak{J}$. *Further,* D_{12}^* *is the transpose of* D_{12} *with respect to the trace form on* $\mathfrak{J}$.

In general, the 133-dimensional space $\mathfrak{L}(\mathfrak{R})$ spanned by the derivations and left multiplications of elements of trace zero in $\mathfrak{R}$ is not closed under the operation of commutation. In fact, we have closure in $\mathfrak{L}(\mathfrak{R})$ if and only if $\delta_1 = \delta_2$, $\mu = 1/3$, $\nu = 1$, and $\omega_1\omega_2 = 4\delta_2$. In this case we will write $\mathfrak{R}$ as $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$, where $\delta = \delta_1 = \delta_2$ and $\omega = {}^1/_2\omega_1$. If $\mathfrak{R} = \mathfrak{R}(\mathfrak{J};\ \delta, \omega)$, then $\mathfrak{L}(\mathfrak{R})$ is a central simple Lie algebra, $\mathfrak{R}$ is an irreducible representation space for $\mathfrak{L}(\mathfrak{R})$, and we have the following theorem.

THEOREM 2. *If the characteristic of the ground field is not two or three, then the derivations and the left multiplications of elements of trace zero in* $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$ *generate an exceptional simple Lie algebra* $\mathfrak{L}(\mathfrak{R})$ *of type* E_7. (This resembles the representation of the Lie algebra $\mathfrak{E}_7$ given by Freudenthal.[3])

We have also computed the automorphism groups of the algebras $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$. This is not difficult, because f_1, f_2, and f are the only idempotents which are annihilated by all derivations of $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$; consequently, if ϕ is an automorphism, then either $f_1\phi = f_1$ and $f_2\phi = f_2$, or $f_1\phi = f_2$ and $f_2\phi = f_1$. Let $L(\mathfrak{J})$ be the group of all nonsingular linear transformations S on $\mathfrak{J}$ such that $N(aS) = N(a)$ for all a in $\mathfrak{J}$, where N is the norm on $\mathfrak{J}$.

THEOREM 3. *If there is a nonsingular linear transformation* P *on* $\mathfrak{J}$ *such that* $N(aP) = \omega^2\delta^{-1}N(a)$ *for all* a *in* $\mathfrak{J}$, *then the automorphisms of* $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$ *are the linear transformations of the form* S': $f_1 \to f_1$, $f_2 \to f_2$, $a_{12} \to (aS)_{12}$ *for* S *in* $L(\mathfrak{J})$, $b_{21} \to (bS^{*-1})_{21}$, *and* $\pi S'$, *where* π: $f_1 \to f_2$, $f_2 \to f_1$, $a_{12} \to (aP)_{21}$, $b_{21} \to (bP^{*-1})_{12}$. *If* P *does not exist, then the automorphisms of* $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$ *are just the group* $\{S' | S \epsilon L(\mathfrak{J})\}$. *However, such a transformation* P *always exists if* $\mathfrak{J}$ *is reduced.*

The structure of the group $L(\mathfrak{J})$ for $\mathfrak{J}$ reduced has been found by Jacobson.[4] He has shown that $L(\mathfrak{J})$ modulo its center is simple and that the center consists of the identity transformation I alone or else of the three transformations I, ϵI, $\epsilon^2 I$, where ϵ is a primitive cube root of unity. This result is essential for the following theorem on the structure of the automorphism groups of the algebras $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$.

THEOREM 4. *Let* $\mathfrak{J}$ *be a reduced exceptional simple Jordan algebra over a field of characteristic not two or three. The automorphism group* Aut $(\mathfrak{R})$ *of* $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$ *has trivial center, and the subgroup* $L(\mathfrak{J})' = \{S' | S \epsilon L(\mathfrak{J})\}$ *is a normal subgroup. The center* $\mathfrak{Z}$ *of* $L(\mathfrak{J})'$ *is a normal subgroup of* Aut $(\mathfrak{R})$ *also and consists of either the identity transformation* I *or of the three transformations* I, ϵI, $\epsilon^2 I$. *Furthermore,* $L(\mathfrak{J})'/\mathfrak{Z}$ *is simple. Finally, if* $\mathfrak{H}$ *is any normal subgroup of* Aut $(\mathfrak{R})$ *containing an element not in* $L(\mathfrak{J})'$, *then* $\mathfrak{H} =$ Aut $(\mathfrak{R})$.

Our final result is a classification of the algebras $\mathfrak{R}(\mathfrak{J};\ \delta, \omega)$ for $\mathfrak{J}$ reduced.

THEOREM 5. *Let* $\mathfrak{J}_1$ *be a reduced exceptional simple Jordan algebra and* $\mathfrak{J}_2$ *be an arbitrary exceptional simple Jordan algebra over a field of characteristic not two or three. Then* $\mathfrak{R}(\mathfrak{J}_1;\ \delta_1, \omega_1)$ *and* $\mathfrak{R}(\mathfrak{J}_2;\ \delta_2, \omega_2)$ *are isomorphic if and only if* $\mathfrak{J}_2$ *is reduced and the coefficient algebras of* $\mathfrak{J}_1$ *and* $\mathfrak{J}_2$ *are isomorphic.*

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THE EULER CLASS AS AN OBSTRUCTION IN THE THEORY OF FOLIATIONS*

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Let X denote an oriented differentiable manifold of dimension n and $\mathfrak{L}$ an oriented foliation[1] of dimension $p < n$. We ask whether there exists an oriented foliation $\mathfrak{L}'$ of X by leaves of dimension $p + 1$ which is *concordant* with $\mathfrak{L}$ in the sense that each p-leaf of $\mathfrak{L}$ lies on a single $(p + 1)$-leaf of $\mathfrak{L}'$. This is clearly a basic question in the theory of foliations, and we shall refer to it as the *first extension problem*. Let us suppose, for the moment, that $\mathfrak{L}$ constitutes an actual fibering of X, so that there exists a base manifold M of dimension $n - p$ and a projection map π: $X \to M$ with $\mathfrak{L} = \{\pi^{-1}(x) : x \in M\}$. It is apparent that an oriented foliation $\mathfrak{L}'$ of X by $(p + 1)$-leaves concordant to $\mathfrak{L}$ is now equivalent to a direction field on M, in which case the topological obstruction to the first extension problem is none other than the Euler class of the tangent bundle to M. For an arbitrary foliation $\mathfrak{L}$, on the other hand, the leaf-space Λ (which results from the identification of all points lying on a single leaf of $\mathfrak{L}$) is usually not a manifold and quite unsuitable to give us an Euler class, since it carries no natural vector bundle. Let us recall at this point that in a previous paper[2] we have introduced a category $\mathcal{C}$ which extends the category C of topological spaces and continuous maps, and we have shown that a foliation $\mathfrak{L}$ canonically determines an object $\mathfrak{X}$ in $\mathcal{C}$ which generalizes the notion of a base manifold. In particular, whenever $\mathfrak{L}$ does admit a classical base manifold M, $\mathfrak{X}$ is naturally isomorphic to M in $\mathcal{C}$. In general, $\mathfrak{X}$ occupies an intermediate position between X and Λ (there exist natural projections $X \to \mathfrak{X} \to \Lambda$), and appears to be a more suitable candidate for the role of a base manifold than the classical leaf-space Λ. This raises the question whether by a suitable development of these ideas it may not be possible to define a notion of *object tangent bundle* to $\mathfrak{X}$ and a corresponding *Euler class* which will constitute an obstruction for the first extension problem. It is the purpose of this note to indicate in outline how this can be done and to state the essential result constituting a general solution to the given problem. For a detailed development of the requisite theory of object fiber bundles and for all proofs we refer to a forthcoming paper. Some geometrical applications of the theory will be considered in the last section of this note.

1. *Object Bundles.*—We recall that an *object* $\mathfrak{X}$ in the category $\mathcal{C}$ was defined[3] to be a pair (X,I), X being a topological space and I a set of local maps u: $X \to X$ (i.e., maps whose domain $D(u)$ is an open subset of X) subject to three axioms: (*i*) I contains the identity map of X; (*ii*) given $u,v \in I$ and $x \in D(u) \cap D(v)$, there shall exist $\hat{u},\hat{v} \in I$ such that $\hat{u} \circ u = v \circ v$ near x (*extension axiom*); (*iii*) given a local map u: $X \to X$ such that for every $x \in D(u)$ there exist $\hat{v},\hat{u},v \in I$ such that $\hat{u} \circ u = \hat{v} \circ v$ near x, then $u \in I$ (*closure axiom*). X will be referred to as the *fundamental space* and

I the set of *fundamental maps* belonging to $\mathfrak{X}$. By a *map* F: $\mathfrak{X} \to \mathfrak{X}'$ between objects, we understand a set of local maps f: $X \to X'$ between their respective fundamental spaces such that, firstly, $\{D(f)$: $f \in F\}$ constitutes a covering of X, and secondly, F satisfies extension and closure axioms (analogous to (*ii*) and (*iii*) above). Given F: $\mathfrak{X} \to \mathfrak{X}'$ and F': $\mathfrak{X}' \to \mathfrak{X}''$, a composite map $F' \circ F$: $\mathfrak{X} \to \mathfrak{X}''$ can be defined by the condition that $F' \circ F$ shall contain all C-maps $f' \circ f$ with $(f,f') \in F \times F'$. It can be verified that these definitions give rise to a category $\mathfrak{C}$. The classical category C of topological spaces and continuous maps can be naturally identified with a full subcategory of $\mathfrak{C}$, and we shall always assume that this has been done. A foliation $\mathfrak{L}$ of X determines an object $\mathfrak{X} = (X,I)$ in $\mathfrak{C}$ (referred to as the *canonical object* of $\mathfrak{L}$), I being a suitably defined[3] set of leaf-preserving maps.

To obtain an adequate notion of tangent vector bundle over $\mathfrak{X}$, one is led to consider the general problem of defining *object* $(\mathfrak{F},\mathfrak{G})$*-bundles* for a given standard fiber $\mathfrak{F}$ and structure group $\mathfrak{G}$ (operating continuously and effectively on $\mathfrak{F}$). A careful study of this question suggests that the full category $C(\mathfrak{F},\mathfrak{G})$ of classical $(\mathfrak{F},\mathfrak{G})$-bundles and corresponding bundle maps does not admit a natural and useful extension in the spirit of our object theory. On the other hand, the smaller category $O(\mathfrak{F},\mathfrak{G})$ of $(\mathfrak{F},\mathfrak{G})$-bundles and *open* bundle maps does admit such an extension, which we now proceed to define.

The following concept will play a basic role: Let f,g be maps in C, and x a point such that $g(f(x))$ is defined. We shall call (g,f) a *proper pair* at x if, for every neighborhood V of x such that $g(f(V))$ is defined, $g^{-1}(g(f(V)))$ is a neighborhood of $f(x)$. It follows that if $h_1gf = h_2gf$ near x (h_i being C-maps), then $h_1g = h_2g$ near $f(x)$. The usefulness of proper pairs derives from this lemma.

An object $\mathfrak{X}$ in $\mathfrak{C}$ will be called *proper* if for every fundamental map u in $\mathfrak{X}$ and $x \in D(u)$ there exists a fundamental map v in $\mathfrak{X}$ such that (v,u) is a proper pair at x. A map F: $\mathfrak{X} \to \mathfrak{X}'$ is said to be *open* if for every $f \in F$ and $x \in D(f)$ there exists a fundamental map u in $\mathfrak{X}'$ such that (u,f) is proper at x.

PROPOSITION 1. *The class of open maps between proper objects constitutes a subcategory of* $\mathfrak{C}$. *Moreover, every* $\mathfrak{C}$*-isomorphism between proper objects is open.*

We shall denote this subcategory by $\mathfrak{O}$ and note that it extends the category O of topological spaces and open maps. It can also be verified that the canonical object of a foliation is always proper.

Let E: $O(\mathfrak{F},\mathfrak{G}) \to 0$ denote the functor which to every bundle assigns its base space. The term *local bundle map* f: $B \to B'$ will be used to denote a bundle map f: $B'' \to B'$, where B'' is an open subbundle of B. An *object bundle* is now defined to be a pair $\mathfrak{B} = (B,J)$, where B is a bundle and J a set of local bundle maps u: $B \to B$ subject to the following axioms: (*i*) the pair $(E(B),I)$, where I denotes the set of all maps $E(u)$ with $u \in J$, constitutes a proper object (referred to as the *base object* of $\mathfrak{B}$ and denoted by $\mathfrak{E}(\mathfrak{B})$); (*ii*) if $u,v,w \in J$ and $E(w) = E(v) \circ E(u)$ near a point $x \in E(B)$, then $w = v \circ u$ near x (i.e., over some neighborhood of x). We shall refer to B as the *fundamental bundle* and J the set of *fundamental maps* of $\mathfrak{B}$.

Let $\mathfrak{B} = (B,J)$ and $\mathfrak{B}' = (B',J')$ be object bundles. A set G of local bundle maps f: $B \to B'$ is said to be an *object bundle map* G: $\mathfrak{B} \to \mathfrak{B}'$ provided: (*i*) $\{E(f)$: $f \in G\}$ constitutes an open map between the base objects (referred to as the *base map* of G and denoted by $\mathfrak{E}(G)$); and (*ii*) if $E(u_1') \circ E(f_1) \circ E(u) = E(u_2') \circ E(f_2)$ near a point $x \in E(B)$, where $u \in J$ and $(f_i,u_i') \in G \times J'$, then $u_1' \circ f_1 \circ u =$

$u_2' \circ f_2$ near x. As in the case of ordinary object maps, one finds that a composite $G' \circ G$ can be defined (G: $\mathfrak{B} \to \mathfrak{B}'$ and G': $\mathfrak{B}' \to \mathfrak{B}''$ being object bundle maps) by the condition that $G' \circ G$ shall contain all (definable) maps $f' \circ f$ with $(ff') \in G \times G'$. It should be pointed out that this circumstance depends strongly on our assumption that the base objects are proper and base maps open.

PROPOSITION 2. *The class of object* $(\mathfrak{F},\mathfrak{G})$*-bundles and corresponding maps, together with the natural law of composition, constitutes a category* (denoted by $\mathcal{O}(\mathfrak{F},\mathfrak{G})$).

We note that $\mathcal{O}(\mathfrak{F},\mathfrak{G})$ extends the classical category $O(\mathfrak{F},\mathfrak{G})$, and the rule $\mathcal{E}$ (defined above) constitutes a functor $\mathcal{E}$: $\mathcal{O}(\mathfrak{F},\mathfrak{G}) \to \mathcal{O}$ extending E. Moreover, the classical functor T: $O(\mathfrak{F},\mathfrak{G}) \to O$, which to every bundle assigns its total space, likewise admits a canonical extension to a functor $\mathfrak{T}$: $\mathcal{O}(\mathfrak{F},\mathfrak{G}) \to \mathcal{O}$. Lastly, we note that the natural transformation π: $T \to E$ representing the projection maps admits a canonical extension to a natural transformation $\Pi: \mathfrak{T} \to \mathcal{E}$.

In connection with our original obstruction problem, we shall be specifically concerned with two kinds of object bundles, viz., *oriented object vector bundles* (where $\mathfrak{F}$ is an oriented vector space and $\mathfrak{G}$ its automorphism group) and *object sphere bundles* (where $\mathfrak{F}$ is a sphere and $\mathfrak{G}$ the proper linear group modulo the positive scalar multiples of the identity). It is easily seen that there exists a natural functor which to every oriented object vector bundle $\mathfrak{B}$ assigns a sphere bundle $\mathfrak{B}_\sigma$ (referred to as the sphere bundle *related* to $\mathfrak{B}$).

Let us now consider a p-dimensional oriented foliation $\mathfrak{L}$ on an n-dimensional oriented manifold X. One may define a quotient B of the tangent bundle over X by annihilating the given distribution of p-planes. B is an oriented vector bundle whose fiber has dimension $n - p$. Let $\mathfrak{X}$ denote the canonical object of $\mathfrak{L}$. We observe that every differentiable fundamental map of $\mathfrak{X}$ admits a unique lifting to B. The totality of these local bundle maps generate an oriented object vector bundle $\mathfrak{B}$ having B for its fundamental bundle and $\mathfrak{X}$ for base object. It will be referred to as the *canonical object vector bundle* of $\mathfrak{L}$. In case $\mathfrak{X}$ is a manifold, $\mathfrak{B}$ is just its tangent bundle. Our obstruction theory will depend upon the following result, which is easily proved:

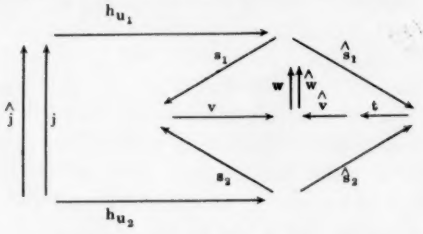
PROPOSITION 3. *A second oriented foliation* $\mathfrak{L}'$ *on* X *of dimension* $p + 1$*, concordant to* $\mathfrak{L}$*, is equivalent to a cross section in the sphere bundle* $\mathfrak{B}_\sigma$ *related to* $\mathfrak{B}$.

2. *Induced Object Bundles.*—Let $\mathfrak{X}$ be a proper object, $\mathfrak{B} = (B,J)$ an object bundle, and F: $\mathfrak{X} \to \mathcal{E}(\mathfrak{B})$ an open map. It is essential to our theory that F induce a bundle $F^{-1}(\mathfrak{B})$ over $\mathfrak{X}$, as in the classical case. The possibility of defining an Euler class depends critically on this circumstance, and it is at this point that one encounters the main difficulty. It may therefore be appropriate for us to consider this question in more detail and thereby to clarify our motivation for some previous definitions.

To define $F^{-1}(\mathfrak{B})$ one must first of all construct its fundamental bundle B_F as a classical $(\mathfrak{F},\mathfrak{G})$-bundle over the fundamental space X of $\mathfrak{X}$. To this end we observe that every local map $u \in F$ brings back the fundamental bundle B of $\mathfrak{B}$ to a bundle B_u over $D(u)$ and defines a bundle map h_u: $B_u \to B$. To obtain a bundle B_F over X, it is obviously needful to identify distinct bundles B_u overlying a common neighborhood of X by means of some natural bundle isomorphisms. Given $(u_1,u_2) \in F \times F$ and $x \in D(u_1) \cap D(u_2)$, there exists $(s_1,s_2) \in J \times J$ (by the map extension axiom) such that $E(s_1) \circ u_1 = E(s_2) \circ u_2$ on some neighborhood U of x.

One can conclude (by classical reasoning) that there exists a unique bundle isomorphism j: $B_{u_1} \to B_{u_2}$ such that (*i*) $E(j)$ is the identity of U, and (*ii*) $s_2 \circ h_{u_2} \circ j = s_1 \circ h_{u_1}$ over U. It is evident that if j should depend on the pair (s_1,s_2), the construction of B_F will not be feasible. We proceed now to establish the uniqueness of j, and it is to be noted that our proof strongly utilizes the concepts of proper object and open map.

Let $(\hat{s}_1,\hat{s}_2) \in J \times J$ be a second pair extending (u_1,u_2) and let $\hat{j}$: $B_{u_1} \to B_{u_2}$ denote the corresponding bundle isomorphism (see diagram).



Since S is an open map, there exists $t \in J$ such that $(E(t), E(\hat{}_2) \circ u_2)$ is a proper pair at x. We conclude by the object extension axiom and axiom (*ii*) for object bundles that there exists $(\hat{v},\hat{v}) \in J \times J$, such that $v \circ s_2 = \hat{v} \circ t \circ \hat{s}_2$ near $u_2(x)$. We conclude similarly that there exists $(w,\hat{w}) \in J \times J$ such that $\hat{w} \circ \hat{v} \circ t \circ \hat{s}_1 = w \circ v \circ s_1$ near $u_1(x)$. It follows by direct calculation that

$$E(\hat{w} \circ \hat{v} \circ t \circ \hat{s}_2) \circ u_2 = E(w \circ \hat{v} \circ t \circ \hat{s}_2) \circ u_2$$

near x. Since $(E(t), E(\hat{s}_2) \circ u_2)$ is proper at x, we may cancel $E(\hat{s}_2) \circ u_2$ on both sides and conclude by axiom (*ii*) for object bundles that $w \circ \hat{v} \circ t = w \circ \hat{v} \circ t$ near $\hat{s}_1(u_1(x))$. By another direct calculation one now verifies that $z \circ j = z \circ \hat{j}$ near x, where $z = w \circ \hat{v} \circ t \circ \hat{s}_1 \circ h_{u_1}$. But this implies $j = \hat{j}$ near x, proving that, for a given pair (u_1,u_2), a canonical isomorphism j is well defined over $D(u_1) \cap D(u_2)$. By a similar argument one can establish that, given three maps $u_1, u_2, u_3 \in F$ having a common domain U, the corresponding isomorphisms j_{ik}: $B_{u_i} \to B_{u_k}$ satisfy the relation $j_{13} = j_{23} \circ j_{12}$. This lemma enables us to construct the fundamental bundle B_F.

Analogous considerations are required to define the set J_F of fundamental maps for $F^{-1}(\mathfrak{B})$, and to establish

PROPOSITION 4. *Given a proper object* $\mathfrak{X}$, *an object bundle* $\mathfrak{B}$, *and an open map* F: $\mathfrak{X} \to \mathcal{E}(\mathfrak{B})$, *there exists an object bundle* $F^{-1}(\mathfrak{B})$ *and a bundle map* H_F: $F^{-1}(\mathfrak{B}) \to \mathfrak{B}$ *such that* $\mathcal{E}(H_F) = F$. *The pair* $(F^{-1}(\mathfrak{B}), H_F)$ *is unique up to natural equivalence. Moreover, given an object map* G: $\mathfrak{X} \to \mathfrak{I}(\mathfrak{B})$ *such that* $\Pi(\mathfrak{B}) \circ G = F$, *there exists a unique cross section* S_G *such that* $G = \mathfrak{I}(H_F) \circ S_G$.

3. *Open Singular Homology.*—The usual definitions of singular homology lead to well-defined homology theories on the various object categories. However, these theories are not suited to our present purpose, owing to the fact that singular simplexes are generally not given by open maps. This means that a singular simplex belonging to the base object of a bundle will fail to induce a bundle over the standard simplex. Since these induced bundles (or something quite equivalent) are essential for the definition of an Euler class, it will be necessary to modify the

basic concepts in such a way that every singular simplex of the new theory is open.

Let H be a separable Hilbert space, $\{\sigma_k\colon\ 0 \leqq k < \infty\}$ a family of standard simplexes, and $\mathfrak{X}$ a proper object. By an *open singular k-simplex* in $\mathfrak{X}$ we shall understand an object map $S\colon\ \sigma_k \times H \to \mathfrak{X}$ such that $S|\{x\} \times H$ is open for all points $x \in \sigma_k$. Let $C_k(\mathfrak{X})$ denote the free abelian group generated by these simplexes. One can define a boundary operator $\partial\colon\ C_k(\mathfrak{X}) \to C_{k-1}(\mathfrak{X})$ as usual and thus obtain a chain complex $\mathcal{S}(\mathfrak{X})$. An open map $F\colon\ \mathfrak{X} \to \mathfrak{X}'$ induces a chain map $F_*\colon\ \mathcal{S}(\mathfrak{X}) \to \mathcal{S}(\mathfrak{X}')$, and we obtain a covariant functor $\mathcal{S}\colon\ \mathcal{O} \to \mathcal{K}$, where $\mathcal{K}$ denotes the category of chain complexes. $\mathcal{S}$ defines a homology theory on $\mathcal{O}$ which may be designated *open singular*.

Let $\mathfrak{B}$ denote an object bundle. By a *basically open singular k-simplex* in $\mathfrak{B}$ we understand a map $S\colon\ \sigma_k \times H \to \mathfrak{I}(\mathfrak{B})$ such that $\Pi(\mathfrak{B}) \circ S|\{x\} \times H$ is open for all points $x \in \sigma_k$. Analogous to what has been said above, these simplexes give rise to a covariant functor $\mathcal{S}_b\colon\ \mathcal{O}(\mathfrak{F},\mathfrak{G}) \to \mathcal{K}$ defining the *open singular homology theory* for object bundles.

Now let $\mathfrak{B}$ denote an oriented object vector bundle ($\mathfrak{F}$ is an oriented n-dimensional vector space) and $\mathfrak{X}$ its base object. To define the Euler class of $\mathfrak{B}$ (as an element in the n-dimensional integral open singular cohomology of $\mathfrak{X}$) we shall require two lemmas. Let $\rho\colon\ \mathcal{S}_b(\mathfrak{B}) \to \mathcal{S}(\mathfrak{X})$ denote the chain map induced by $\Pi(\mathfrak{B})$, and $\omega\colon\ \mathcal{S}(\mathfrak{X}) \to \mathcal{S}_b(\mathfrak{B})$ the chain map induced by the zero cross section of $\mathfrak{B}$ (defined in a natural way).

LEMMA 1. *(ρ,ω) constitutes an equivalence pair* (i.e., both composites are chain homotopic to the identity).

Given a basically open simplex S in $\mathfrak{B}$, we note that $\Pi(\mathfrak{B}) \circ S$ brings $\mathfrak{B}$ back to a bundle B_S over $\sigma_k \times H$, and S determines a cross section f_S in B_S. We shall say that S lies *away from zero* if f_S does (which makes sense because B_S and f_S are classical). Let $\mathcal{S}_0(\mathfrak{B})$ denote the subcomplex of $\mathcal{S}(\mathfrak{B})$ generated by singular simplexes whose $(n-1)$-skeletons lie away from zero, and let $\mu\colon\ \mathcal{S}_0(\mathfrak{B}) \to \mathcal{S}_b(\mathfrak{B})$ denote the inclusion map.

LEMMA 2. *There exists a chain map $\tau\colon\ \mathcal{S}_b(\mathfrak{B}) \to \mathcal{S}_0(\mathfrak{B})$, such that (μ,τ) is an equivalence pair.*

Let S be a generating n-simplex of $\mathcal{S}_c(\mathfrak{B})$. As noted above, S determines a cross section $f_S\colon\ \sigma_n \times H \to B_S$. Let $p\colon\ B_S \to \mathfrak{F}$ denote the projection map associated with a product representation of B_S. As in the classical theory, one observes that $p \circ f_S|\sigma_n \times \{0\}$ determines a relative n-cycle in $C_n(\mathfrak{F}, \mathfrak{F}_0)$ (where $\mathfrak{F}_0$ denotes the punctured vector space) which in turn determines an element z in the nth relative integral homology group. Since the latter is infinite cyclic and has a preferred generator z_1, we may define an integer λ by the equation $z = \lambda z_1$. It is easy to verify that λ is independent of p and defines a cocycle in $\mathcal{S}_0(\mathfrak{B})$. This gives us an element in the nth integral cohomology of $\mathcal{S}_0(\mathfrak{B})$, which can be brought down (by Lemmas 1 and 2) to an element in the corresponding cohomology group of $\mathfrak{X}$. This will be called the Euler class of $\mathfrak{B}$. It may be noted that, given an object bundle map $G\colon\ \mathfrak{B} \to \mathfrak{B}'$, the base map $\mathcal{E}(G)$ brings back the Euler class, as in the classical theory.

We may now state our main result:

THEOREM. *The Euler class of $\mathfrak{B}$ constitutes an obstruction to the existence of cross sections in the sphere bundle related to $\mathfrak{B}$.*

COROLLARY. *Let X be an oriented differentiable manifold and $\mathfrak{L}$ an oriented folia-*

tion of X. The Euler class of the canonical object vector bundle of $\mathfrak{L}$ constitutes an obstruction for the first extension problem.

4. *Geometrical Applications.*—We will now consider the first extension problem in the simplest case, i.e., where X is three-dimensional Euclidean space and $\mathfrak{L}$ a foliation of X by curves. Since every foliation of X is now orientable, our question reduces to this: does there exist a foliation $\mathfrak{L}'$ of X by surfaces concordant to $\mathfrak{L}$? One may expect that the obstruction to $\mathfrak{L}'$ would be due mainly to closed curves in $\mathfrak{L}$. It will be of interest to consider the following notion of a *toroidal family:* Let T denote a solid torus, which we may represent as a subset of (x,y,z)-space given by $x^2 + y^2 \leqq 1$ with the understanding that z is defined mod 1. The lines parallel to the z-axis define a foliation $\mathfrak{L}_0$ of T. We will say that a given set τ of curves in $\mathfrak{L}$ constitutes a *toroidal family* if there exists a homeomorphism ϕ: $T \to X$ taking $\mathfrak{L}_0$ into τ. Having chosen an orientation for X and $\mathfrak{L}$, one can assign to every toroidal family two integer-valued indexes as follows. The first is just the linking coefficient of neighboring curves in τ, and will be denoted by $l(\tau)$. In other words, $l(\tau)$ equals the number of full twists of the imbedded torus. The second index (which we denote by $\chi(\tau)$) is closely related to our Euler class and depends not just on τ but on the entire foliation $\mathfrak{L}$. Its definition depends on the fact that one can associate with τ a canonical homology class h in the 2-dimensional integral homology of $\mathfrak{X}$ (the canonical object of $\mathfrak{L}$). Roughly speaking, h is represented by a surface in X bounded by a curve in τ. We define $\chi(\tau)$ to be the period of the Euler class on h. It is rather easy to compute, and by our theorem constitutes an obstruction for the extension problem.

The connection between the two indexes may be clarified as follows: *the parity of $\chi(\tau)$ is always opposite to that of $l(\tau)$. This relation is maximal in the sense that all pairs $(\chi(\tau), l(\tau))$ with opposite parity are actually realizable.* It follows therefore as a corollary to our obstruction theorem that the existence of a solution $\mathfrak{L}'$ to the extension problem would imply that $l(\tau)$ is odd. It is interesting to note that this corollary may be established directly by the following classical argument: given X, $\mathfrak{L}$, and $\mathfrak{L}'$ (all oriented), one may associate with every point $x \in X$ a proper orthogonal reference frame in a natural way (the first vector being taken tangent to the given $\mathfrak{L}$-curve, the second tangent to the $\mathfrak{L}'$-surface). This frame field enables one to define a map ψ: $X \to O_3$ (the proper orthogonal group), and one finds that a toroidal family τ determines now an element α in the fundamental group $\pi_1(O_3)$. Under the given hypothesis, α must be the zero element, and one verifies by direct calculation that this is equivalent to the condition that $l(\tau)$ be odd.

As a last observation concerning this given geometric problem, we remark that there does exist a foliation $\mathfrak{L}$ with a toroidal family τ such that (*i*) $\chi(\tau) = 0$, (*ii*) $l(\tau) = 1$, and (*iii*) the extension problem admits a solution.

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[1] By a *foliation of dimension p* we understand an involutive distribution of p-planes. Its *leaves* are the maximal integral submanifolds of the distribution. Cf. Chevalley, C., *Theory of Lie Groups*, I (Princeton University Press, 1946).

[2] Clifton, Y. H., and J. W. Smith, "The category of topological objects," these PROCEEDINGS, **47**, 190–195 (1961). A detailed account of this theory in sheaf language may be found in their article "Topological objects and sheaves," *Trans. Amer. Math. Soc.*, **105**, 436–452 (1962).

[3] *Ibid.*, section 5.

*ANTIBIOTIC GLYCOSIDES, IV. STUDIES ON THE MECHANISM OF ERYTHROMYCIN RESISTANCE IN BACILLUS SUBTILIS**

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Three general patterns for the acquisition of antibiotic resistance have been described in microorganisms.[1] These bacterial modifications include: (*a*) the development of a mechanism for the complete, or an increased rate of, destruction of the antibiotic, (*b*) a decreased or complete loss of permeability of the cell for the antibiotic, and (*c*) the acquisition or enhancement of a mechanism for overcoming the antibiotic-inhibited process. Examples of each type of resistance have been reported from studies of microorganisms.[2–10] Only the last of the above-mentioned processes need be related to the mechanism of action of the antibiotic. One or more of these mechanisms, or a hitherto unrecognized type of alteration in cellular physiology, may be operative in the development of antibiotic resistance in a specific microorganism. In the case of erythromycin-A,[11] one of the macrolide group of antibiotic glycosides, there has been no convincing confirmation or rejection of any of the mechanisms of resistance mentioned. Although qualitative recovery of antibiotic activity, after growth of resistant organisms in the presence of erythromycin, has been reported,[12, 13] no attempt was made to demonstrate either the retention of the original chemical structure or the fate of any intracellular antibiotic. Relative to mechanism (*c*), Brock and Brock have described an inhibition of protein synthesis by both chloramphenicol and erythromycin in *Escherichia coli*.[14] However, because this organism is not very sensitive to erythromycin, a very high concentration of the antibiotic (1 mg/ml) was used. No data relative to the effect of such a high antibiotic concentration on protein synthesis in a less sensitive strain of *E. coli* were presented.

In order to extend the previous observations, and to confirm their validity for a microorganism sensitive to therapeutic levels of erythromycin (<1 μg/ml), a study was undertaken to investigate the mechanism of action of erythromycin and to explore the biochemical basis of resistance to erythromycin in *Bacillus subtilis*. Results with an erythromycin-resistant, genetically stable mutant were compared with those obtained with the erythromycin-sensitive parent strain. The data obtained demonstrate that neither degradation of the antibiotic nor change in permeability to the antibiotic play significant roles in resistance. Erythromycin appears to interfere with some aspect of the utilization of amino acids by the sensitive strain of *B. subtilis*. This same process is not significantly inhibited, at comparable antibiotic levels, in the erythromycin-resistant strain.

Experimental.—Bacillus subtilis: Erythromycin-resistant mutants of *B. subtilis* were obtained by plating organisms (1×10^8) of the wild strain (*B. subtilis* 23) and an indole auxotroph (*B. subtilis* 168) on minimal glucose agar[15] containing erythromycin (2 μg/ml, 2.7 μM). The genetic nature of the erythromycin resistance was demonstrated by transformation of the erythromycin-sensitive strain to resistance by preparations of DNA from the erythromycin-resistant strain.[16] An additional genetic marker, adenine auxotrophy, was introduced by transformation of the strains by DNA from a mutant strain of *B. subtilis* 168 (SB 23).[17] Thus, organisms utilized in this in-

TABLE 1

CHARACTERISTICS OF BACTERIAL STRAINS

Strain designation	Genetic traits	Erythromycin ID_{50}* μg/ml	Growth requirements
Erythromycin-resistant	I^-, Ad^-, Ery^R	3.0	Indole, adenine
Erythromycin-sensitive	I^-, Ad^-, Ery^S	0.04	Indole, adenine

* ID_{50} = concentration required to inhibit growth 50% starting with an inoculum of about 10^6 bacteria per ml.

vestigation have the designations, genetic properties, and erythromycin sensitivities shown in Table 1.

Stock cultures of *B. subtilis* were maintained as described previously.[18] Bacteria were first grown in minimal salts medium[16] containing glucose (0.5%), neutralized acid hydrolyzed casein (0.02%), L-tryptophan (50 μg/ml), and adenine hydrochloride (50 μg/ml). All experiments were carried out at 37°C with logarithmically growing cells in the above medium on a New Brunswick rotary shaker, either model WBR at 200 rpm, 1/2" stroke, or Model G-25 at 300 rpm, 1" stroke.

Turbidities were determined on a Klett-Summerson Colorimeter (filter number 54) and correlated with viable cell count (one Klett unit is approximately equivalent to 3×10^6 bacteria/ml).

Preparation of H^3-erythromycin: Erythromycin-A was synthesized by *Streptomyces erythreus*, utilizing sodium 2,3-H^3-propionate (Volk Radiochemicals, Inc.) as a precursor. Preliminary isolation of the erythromycin synthesized was as described.[19] The final purification was by partition chromatography at 25°C on a powdered cellulose column (34 × 1.8 cm, 50 *g*) employing the lower layer of a methylcyclohexane:n-butanol:acetone:phosphate buffer (pH 6.85, 0.01 *M*) system (75:15:10:25 v/v) as the stationary phase (2:1 w/v) and the upper layer of the same system as the mobile phase. The effluent fractions containing the bulk of the antibiotic activity and radioactivity were found to correspond exactly. Solute isolated from these fractions gave only a single radioactive spot (corresponding to authentic erythromycin-A) on paper chromatography, using the above solvent system and also using a modification of that system.[20] Both of these systems have been shown capable of separating erythromycin-A from closely related compounds, some of which have antibiotic activity. Radioactive purity of the H^3-erythromycin was further indicated by retention of the activity on dilution with nonradioactive erythromycin, followed by chemical derivatization and purification as erythromycin-2"-O-benzoate.[19] The specific activity of the H^3-erythromycin obtained from this preparation was 2×10^4 cpm per μg.

Recovery of H^3-erythromycin from cultures of B. subtilis: Experiments were carried out in which *B. subtilis* was grown with H^3-erythromycin (20,000 cpm/μg) added to the standard minimal salts medium. After the desired level of growth was attained, each incubation was terminated by cooling to 0°C. In the experiments from which both extra- and intracellular erythromycin were recovered, a large amount of nonradioactive erythromycin (25 mg) was added, after which the bacteria were lysed with lysozyme and the erythromycin fraction was isolated by chloroform extraction at pH 9.5.[19] In the experiment from which only intracellular erythromycin was recovered, the bacteria (approximately 7.5×10^{10} organisms) were first washed (five times, 10 ml each time) with minimal salts medium containing glucose (0.5%) and nonradioactive erythromycin (10 μg/ml). Finally, a large amount of nonradioactive erythromycin (25 mg) was added, after which the bacteria were lysed with lysozyme and the antibiotic was recovered by diethyl ether extraction at neutral pH. In both cases, recovery of H^3-erythromycin was calculated by the isotope dilution method, after the preparation, isolation, and purification of erythromycin-2"-O-benzoate.[19]

Studies of H^3-erythromycin uptake by B. subtilis: The uptake of H^3-erythromycin from the extracellular medium, by erythromycin-sensitive and erythromycin-resistant bacteria, was determined by filtering an aliquot of the bacterial suspension through a membrane filter (Millipore, Type HA, 25 mm). The bacteria collected in this manner (about 5×10^8 organisms) were then quickly washed by repeated suspension in the filtration apparatus (seven times, 2.5 ml each time) in minimal salts medium containing glucose (0.5%) and nonradioactive erythromycin (10 μg/ml). After washing, the bacteria and the membrane filter were dried by gentle heating (<40°C) and then added to a liquid scintillation counting vial containing 10 ml of the toluene-based scintillant described by Davidson.[21] The toluene, besides lysing the bacteria, permeates the pores of the membrane filter and renders it almost completely transparent.

Lysozyme-spheroplasts (osmotically sensitive), prepared according to the method of Landman,[22] were used to determine the role of the intact cell wall of *B. subtilis* in erythromycin uptake. After collection by gentle filtration, the spheroplasts were washed (as described above) with a solution containing sodium succinate (0.5 *M*), $MgSO_4$ (0.01 *M*), and EDTA (0.005 *M*).

Studies of H^3-tryptophan and C^{14}-adenine uptake by B. subtilis: The experimental techniques used were essentially the same as outlined above for the studies of H^3-erythromycin uptake, except that the nonradioactive forms of the compounds being studied were added to the resuspending solution. The C^{14}-adenine hydrochloride (0.02 μC/mg) and the H^3-tryptophan (4.0 μC/mg) were obtained from Calbiochem and Volk Radiochemicals, Inc., respectively.

Results and Discussion.—The initial experiments with the erythromycin-resistant strain of *B. subtilis* showed that 90 per cent of the erythromycin which was added to cultures of the bacteria could be recovered after growth (Table 2, flask 1). From 94–97 per cent of added erythromycin was recovered from two control experiments, one of which employed the erythromycin-sensitive strain of *B. subtilis* (Table 2, flask 2) and the other of which received no bacterial inoculum (Table 2, flask 3). In these experiments the antibiotic was recovered from the combined medium and the lysozyme-lysed bacteria. In a different experiment the resistant bacteria, after growth in the presence of tritiated erythromycin, were washed repeatedly to remove adsorbed H^3-erythromycin and then lysed with lysozyme. Here it was possible to recover 100 per cent of the radioactivity taken up by the bacteria. In this case the external erythromycin concentration was 0.5 μg per ml (2×10^4 cpm/μg) and the uptake of erythromycin was 0.035 μg per 10^{11} bacteria. Collectively, these experiments rule out the possibility that antibiotic degradation plays a significant role in the mechanism by which the erythromycin-resistant mutant strain of *B. subtilis* grows in the presence of normally bacteriostatic concentrations of erythromycin. The results are in agreement with the qualitative findings of both Haight and Finland[12] and Straightoff,[13] who investigated this same problem by means of a procedure similar to that developed by Gots[23] to detect penicillinase activity. Since loss of extracellular biological activity served as the sole criterion of antibiotic destruction in these studies, subtle chemical change would not have been recognized unless it led to marked diminuition of antibiotic activity. Furthermore, these experiments, as well as those reported in Table 2, did not test the possibility that the mutant, resistant bacteria have acquired some mechanism for the intracellular destruction of erythromycin prior to its reaching the normal site of antibacterial action. The fact that gross intracellular destruction of erythromycin does not occur is demonstrated by the result of the experiment in which the erythromycin taken up by the resistant bacteria was quantitatively recovered.

The recovery of unchanged erythromycin from the resistant, mutant forms of *B. subtilis* does not rule out a change in permeability of the bacterial cell for the

TABLE 2

RECOVERY OF H^3-ERYTHROMYCIN FROM CULTURES OF *Bacillus subtilis*

	Flask No. 1	Flask No. 2	Flask No. 3
Bacteria	Erythromycin-resistant	Erythromycin-sensitive	None
Inoculum (cells/ml)	1.1×10^6	1.6×10^6	—
Growth, (Δ Klett-units) (filter no. 54)	122	3	—
Total H^3-erythromycin recovered, (μg)	10.75	11.3	11.6
Recovery as erythromycin-2″-O-benzoate (%)	89.5	94.2	96.8

Incubations were carried out in 300-ml nephelometer flasks with 15 ml of the standard minimal medium containing 0.8 μg H^3-erythromycin per ml.

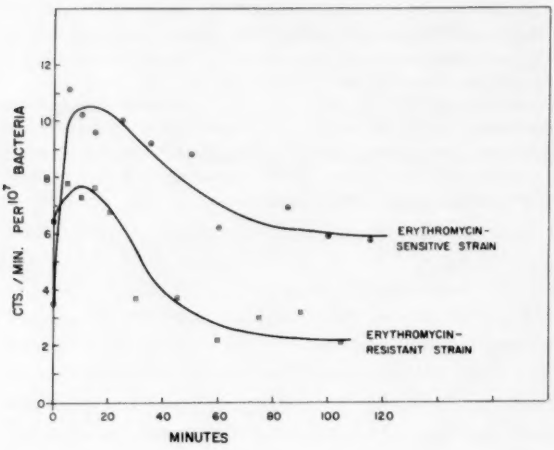

FIG. 1.—Uptake with time of erythromycin by erythromycin-sensitive and erythromycin-resistant *B. subtilis*. At the start of the experiment the bacteria (approximately 5×10^7 per ml) were present in standard minimal medium (20 ml) containing H^3-erythromycin (0.5 μg/ml, 2×10^4 cpm/μg). The incubations were carried out in 300-ml nephelometer flasks, aliquots were removed at varying time intervals, the bacteria were collected and washed, and the uptake of H^3-erythromycin was determined as described in the text.

antibiotic as being an essential part of the resistance mechanism. Accordingly, several experiments were carried out to investigate the possible role of antibiotic exclusion in the resistance to erythromycin observed in *B. subtilis*. Although a difference in uptake of H^3-erythromycin, between the sensitive and resistant bacteria, was consistently observed in these experiments (Figs. 1 and 2) it was much lower than the 100-fold difference in their sensitivity (Table 1). A similar difference in the uptake of antibiotic also was noted in lysozyme-spheroplasts of the sensitive and resistant strains. The data presented graphically in Figure 1 indicate that there is an initial rapid uptake of the antibiotic and then a slower release until an equilibrium level is attained. The amount of antibiotic actually taken up from the extracellular medium is insignificant in terms of lowering the concentration of antibiotic present. We can offer no logical explanation for the complex uptake phenomenon represented in Figure 1.

With increasing extracellular concentrations of the antibiotic, the uptake of H^3-erythromycin appears to increase proportionately in both the sensitive and resistant strains of *B. subtilis* (Fig. 2). This occurs in spite of the fact that cell multiplication ceases in the erythromycin-sensitive strain when an antibiotic level of 7.5 μg per 10^{11} bacteria is reached. Using the data presented in Figure 2, and the approximate size of the bacteria, one can calculate[24] both the internal concentration of the antibiotic (assuming homogeneous dispersion) and the degree of its concentration or dilution as compared with that in the extracellular fluid. The erythromycin-sensitive strain can effect an antibiotic concentration of about 60-fold, while the resistant organism concentrates the drug only 20 times above the extracellular level. At the higher antibiotic concentrations, as represented in Figure 2, the uptake of erythromycin per bacterium for the resistant strain has already surpassed the level which inhibits multiplication in the sensitive strain ($\geqq$7.5 μg erythromycin/10^{11} bacteria). In spite of this there is no effect on the multiplication rate of the resistant bacteria. This provides a further indication that a loss of permeability cannot explain the observed resistance. It is, of course, possible that the two strains of *B. subtilis* differ somewhat in intracellular distribu-

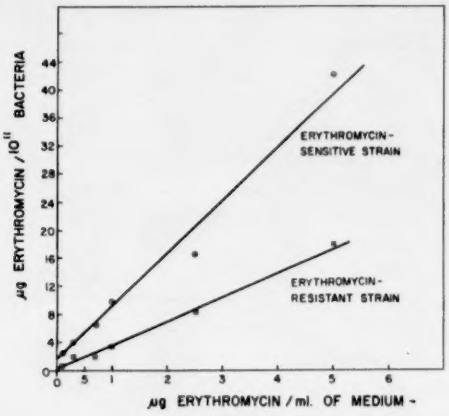

FIG. 2.—Uptake of erythromycin by erythromycin-sensitive and erythromycin-resistant *B. subtilis* as a function of extracellular antibiotic concentration. The bacteria ($2–5 \times 10^8$) were added to standard medium (5 ml) in an Erlenmeyer flask (25 ml) containing varying concentrations of erythromycin. Depending on the concentration, the specific activity of the erythromycin varied from 2×10^4 to 10^5 cpm per μg. The cells were incubated at 37°C on a rotary shaker. After 60 min an aliquot was withdrawn, the bacteria were collected and washed, and H^3-erythromycin uptake was determined as described in the text. The remainder of the sample was diluted and used for a viable cell count.

tion of the antibiotic. If so, this, coupled with the difference in uptake which is observed (Fig. 1), might result in a substantial difference in the amount of erythromycin which reaches the sensitive site within the bacterium. Results to be presented below minimize this possibility by showing that some other mechanism for the resistance does in fact exist.

In the case of *B. subtilis* there is a rapid exchange between intra- and extracellular erythromycin. The data showing this, in the case of the sensitive strain, are plotted in Figure 3. Similar results were obtained using the erythromycin-resistant strain, although the accuracy was below that obtained with the sensitive strain because a smaller amount of antibiotic was taken up. The almost complete recovery of intact erythromycin from the combined medium and bacteria of *B. subtilis* cultures (Table 2) is particularly significant in view of this rapid and reversible uptake of the antibiotic by the bacterial cells. It seems likely that much of the H^3-erythromycin reisolated from the medium in these experiments had been "taken up" by the bacteria, only to be released again unchanged. If intracellular destruction could occur, then the extracellular erythromycin should have been gradually diluted with the degradation products, if these could be released by the bacteria. If they could not be released, the extracellular pool of erythromycin should have decreased in size. Since neither of these effects was noted, the recovery of erythromycin from the medium in these experiments is significant in supporting the conclusion that erythromycin is not destroyed intracellularly by the resistant bacteria.

An approximation of the over-all processes of protein and nucleic acid synthesis in the erythromycin-resistant and -sensitive strains of *B. subtilis* was possible because of the requirements of these organisms for exogenous L-tryptophan and adenine. Studies of the utilization of these two substrates were carried out in both the presence and the absence of erythromycin (1 μg/ml). With erythromycin present, the resistant mutant grows at an essentially unaltered rate, while growth of the sensitive strain is reduced to about 20 per cent of the normal rate (as measured turbidimetrically). The data obtained from these studies are presented in Figures 4*A–D*. They were obtained by measuring both the turbidity and bacterial radio-

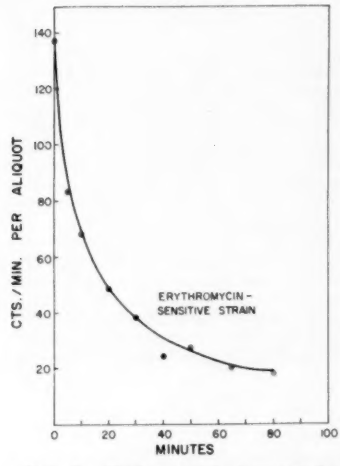

FIG. 3.—Exchange of intracellular and extracellular erythromycin on incubation of *B. subtilis*. The erythromycin-sensitive strain was inoculated into the standard minimal medium containing H^3-erythromycin (0.1 μg/ml, 2×10^4 cpm/μg), and incubated for 60 min at 37°C with shaking. The bacteria were then separated from the medium by centrifugation and resuspended (5×10^8 bacteria/ml) in standard minimal medium containing nonradioactive erythromycin (0.1 μg/ml). Aliquots of the suspension were removed at varying time intervals, after which the bacteria were collected by filtration and washed, and the content of H^3-erythromycin was determined as described in the experimental portion of the text.

activity of aliquots of the suspensions of *B. subtilis* at varying time intervals. These data show that uptake of H^3-tryptophan and C^{14}-adenine was similar in both the erythromycin-resistant and the erythromycin-sensitive strain of *B. subtilis*, as long as erythromycin was absent (Figs. 4*A* and 4*C*, respectively). In both of these cases the ratio of incorporation of adenine relative to tryptophan was about 1.8:1. When erythromycin was present, however, a marked difference between the two strains of *B. subtilis* was observed. The resistant strain grew at almost a normal rate, and both adenine and tryptophan were incorporated in about the same proportions as before (Fig. 4*B*). The erythromycin-sensitive strain grew at a low rate, and a reduced but significant incorporation of adenine occurred. Tryptophan incorporation, however, was disproportionately low (Fig. 4*D*). The ratio of incorporation of adenine relative to tryptophan was increased (3.2:1), and this strongly suggests that some aspect of the mechanism whereby amino acids are utilized for protein synthesis is affected by erythromycin in the sensitive strain of *B. subtilis*. The resistant mutant is able to utilize amino acids at nearly the normal rate in the presence of the antibiotic.

So and Davie,[25] using a cell-free amino acid incorporation system derived from yeast and *E. coli*, have recently studied some of the characteristics of chloramphenicol action. In collaboration with these authors, and using a similar cell-free system derived from the above strains of *B. subtilis*, we have been able to demonstrate that the incorporation of amino acids (lysine, valine, leucine), by extracts from the sensitive cells is markedly inhibited by erythromycin, while the incorporation is only slightly inhibited in extracts from the resistant cells.[26]

The results obtained with the cell-free system, besides demonstrating the existence of a method for further study of the mechanism of erythromycin action, increase the probability that resistance to the antibiotic in *B. subtilis* is related to the effect of erythromycin on amino acid utilization. The possibility that resistance results from a difference in intracellular distribution of the erythromycin (see above) between the resistant and sensitive forms of the *B. subtilis* seems unlikely, since a difference in sensitivity to the antibiotic is maintained in the cell-free state.

The findings presented in this communication provide strong evidence that the mechanism of resistance of a *B. subtilis* mutant to erythromycin is one whereby an increased tolerance to the antibiotic-inhibited process has developed. The

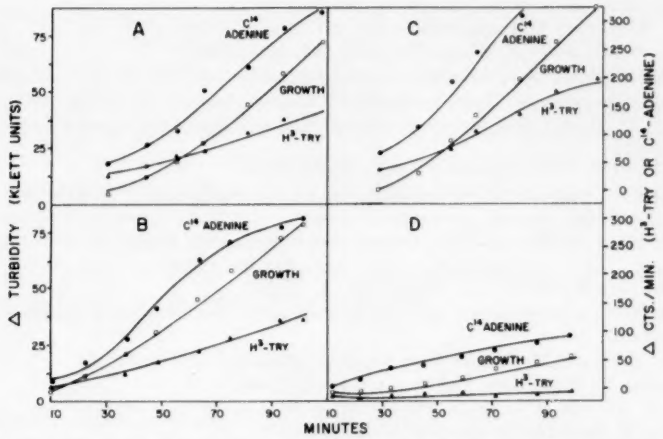

FIG. 4.—The effect of erythromycin on the uptake of C^{14}-adenine and H^3-tryptophan by erythromycin-sensitive and erythromycin-resistant *B. subtilis*. At the start of the incubation the bacteria (~10^8/ml) were suspended in standard minimal salts medium containing glucose (0.5%), neutralized acid hydrolyzed casein (0.02%), H^3-L-tryptophan (5 μg/ml, 4 μC/mg), and 8-C^{14}-adenine hydrochloride (50 μg/ml, 0.02 μC/mg). Logarithmically growing cells from the flasks growing without erythromycin were used to inoculate the flasks containing erythromycin. Aliquots of each bacterial incubation were removed at varying time intervals, the bacteria collected by filtration and washed, and the uptake of the radioactive constituents was determined as described in the text. *A*—erythromycin-resistant *B. subtilis*, without erythromycin. *B*—erythromycin-resistant *B. subtilis*, with erythromycin (1 μg/ml). *C*—erythromycin-sensitive *B. subtilis*, without erythromycin. *D*—erythromycin-sensitive *B. subtilis* with erythromycin (1 μg/ml).

erythromycin seems to interfere with some aspect of amino acid utilization in sensitive bacteria.

Summary.—Erythromycin resistance has been studied in a resistant, genetically stable strain of *B. subtilis* and compared with the erythromycin-sensitive parent strain. Destruction of the antibiotic has been ruled out as a significant factor in this resistance, and exclusion of the antibiotic also does not appear to account for the change in erythromycin sensitivity. Using the auxotrophic requirements (L-tryptophan and adenine), it has been demonstrated that the utilization of amino acids is inhibited by erythromycin in the sensitive bacteria and that the resistant cells are able to overcome this inhibition. Thus, the mechanism of resistance seems to be one in which an increased tolerance to the antibiotic-inhibited process has occurred.

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* For paper III in this series see reference 19.

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*DELOCALIZED VERSUS LOCALIZED PICTURES IN RESONANCE ENERGY TRANSFER**

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1. *Introduction.*—At the conference on Comparative Effects of Radiation the question was discussed whether in excitation transfer a delocalized (collective) or a localized picture should be accepted.[1] The consensus of participants appears to have been that a delocalized picture is appropriate for strong and medium interac-

tions;[2] but that the weak interaction case must be treated in a localized way, such that ". . . the excitation is temporarily entirely localized in one single molecule."[3] This distinction was based on the presence of coherence in strong and medium interactions, and the absence of coherence in the weak-coupling case.

We are going to show that the criterion to be applied is not the presence or absence of coherence, but the presence or absence of localizing processes in the system.[4] As a consequence of this criterion, we find that for weak interactions in liquid solutions, the prototype of localization in past treatments, the general rule is delocalization.

2. *Localization Criterion.*—In the treatment of energy transfer in a system of N identical molecules by the Schrödinger equation,

$$(H_o + H')\Psi = i\hbar \frac{\partial \Psi}{\partial t}, \tag{1}$$

H_o is the Hamiltonian of the unperturbed system, and H' that of the interaction. We first consider H' to result only from resonance interactions among pigment molecules. The solution is written as a superposition of localized wave functions, ϕ_k, such that

$$\Psi = \sum_{k=1}^{N} a_k(t)\phi_k, \tag{2}$$

where ϕ_k is a product,

$$\phi_k = u_1 \ldots u_k^* \ldots u_N, \tag{3}$$

of the wave functions, u_l, of the individual molecules. The symbol * indicates the wave function of the excited state.

In (2), the a_k's are (continuous) functions of time, and are generally nonzero,[5] even if initially only one of them is different from zero. This expresses the fact that initially *localized* excitations become delocalized in time; localized states are not stationary. Therefore, the extreme localization theory, in which the energy resides in one molecule until the location of the energy changes suddenly to another molecule, does not follow from the above quantum mechanical treatment.

The Hamiltonian should include interactions between pigment and solvent molecules in the case of a solution. Inclusion of these interactions makes the form of (1) very complicated; still, in principle, the a_k's are generally nonzero, and the delocalized picture applies.

On the other hand, one can look for self-contained *measuring* processes which localize the excitation energy. The results of such measuring processes are different values of a parameter q, dependent on the energy of a pigment molecule. If

$$|q(E^*) - q(E)| > \delta q, \tag{4}$$

then the outcome of a particular measurement distinguishes E^* from E in a single molecule. Here, E^* and E are the energies of the excited and ground states, respectively, and δq is the quantum mechanical uncertainty of q under the prevailing circumstances.

As an example, consider the absorption of a blue quantum and subsequent emission of a red quantum. If a radiationless transition of the molecule takes place

from the higher to the lower excited state, energy is given to the surroundings. If this relaxation energy is larger than the energy uncertainty of the surroundings, then the red quantum energy is initially localized to one molecule.

In the following, we examine three processes which previously have been considered causes of localization in weak interaction energy transfer in liquid solutions.

(a) *Elastic collisions between pigment and solvent molecules:* Nonquenching collisions produce random phase changes in the oscillations of dipoles. The resulting lack of coherence has been interpreted as the main cause of, or even equivalent to, localization.[6]

We show that phase-changing collisions in liquids are not, in general, energy measuring processes. The quantity q of (4) is here the momentum of the scattered particles. The prototype of such energy measurements is the Stern-Gerlach experiment. The scattering then takes place in a well-prepared field of constant gradient into which a properly defined wave packet of the molecule enters. The momentum imparted to the molecule while it travels through the field is $(\partial E^*/\partial x)T$ when the molecule is excited and $(\partial E/\partial x)T$ in the ground state.[7] T is the duration of the experiment. The experiment is successful if

$$\Delta p_x = \left(\frac{\partial E^*}{\partial x} - \frac{\partial E}{\partial x}\right) T > \delta p_x, \tag{5}$$

where δp_x is the uncertainty of the momentum in the previously prepared wave packet; i.e., $\delta p_x \sim h/d = (\lambda/d)p$, where λ is the de Broglie wavelength of the particle and $d = \delta x$ is the width of the beam. The gradient $\partial E/\partial x$ is independent of x in such experiments. Since $\partial E/\partial x$ increases with applied field strength, it is in principle always possible to satisfy condition (5).

While the molecule passes through the field, its oscillation frequency,

$$\nu = h^{-1}[E^*(F) - E(F)], \tag{6}$$

is a function of the field strength F. In the time interval T a phase change

$$\Delta\varphi = 2\pi h^{-1}[E^*(F) - E(F) - h\nu_0]T \tag{7}$$

is produced; ν_0 is the frequency in the absence of the field.

It is important to note that while Δp_x in (5) depends on the gradient of the applied field, $\Delta\varphi$ depends on the field strength itself. Therefore, the change in the momentum (which is utilized for the energy measurement) and the change in phase produced in the experiment are in general uncorrelated quantities.[8] A simple example is a uniform field $(\partial F/\partial x = 0)$ which produces $\Delta\varphi$ but no Δp_x. This leads one to suspect that phase changes by collisions (and hence destruction of coherence) do not necessarily effect localization.

The collisions between two molecules (a pigment and a solvent) in a liquid represent quite a departure from the well-prepared conditions of a Stern-Gerlach experiment. In the former, the uncertainties of the position and momentum of *both* colliding partners enter the initial conditions, and the acting forces and their gradients change violently with the distance (impact parameter). If the position uncertainty of the two molecules is larger than the range of the forces, then the wave packets corresponding to E and E^* are not resolved with respect to space coordinates. Therefore, it is impossible to decide between E and E^* in a single experiment.

Estimates given below show that this is the case in a liquid at room temperature.

The average de Broglie wavelength at room temperature for a molecule of molecular weight 100 is $\lambda \sim 10^{-9}$ cm. A mean "free path" of the molecule can be estimated from its thermal velocity and the collision frequency. This latter quantity, ν_c, as determined from the spectral broadening, is $\nu_c \sim 10^{12}$ to 10^{13} sec^{-1}. The ratio of the mean free path, Λ, to the de Broglie wavelength is $\Lambda/\lambda \sim 10$ for $\nu_c = 10^{12}$ and $\Lambda/\lambda \sim 1$ for $\nu_c = 10^{13}$ sec^{-1}. The smallest position uncertainty of the colliding molecules is λ; if this is the case the directions of the momenta are completely uncertain and no knowledge of the energy E^* or E can be gained from the scattering experiment.

On the other hand, if the position uncertainty is as large as its maximum, Λ, then the impact parameter is unknown to within Λ. Since in the collision short-range forces (van der Waals) are certainly also involved, the outcome of the individual experiment can be the same angle of scattering for both E and E^*.

Therefore, phase disturbing collisions do not in general localize the excitation. Very infrequently, a Λ large enough and a λ small enough to distinguish E^* from E may occur, but by no means are such events the rule.

(b) *Exchange of vibrational energy:* If v labels vibrational levels in both ground and excited states of the molecule, then $(E_v - E_{v'})$ may differ from $(E_v^* - E_{v'}^*)$. To distinguish E^* from E when energy exchange with the surroundings occurs,

$$\left|(E_v^* - E_{v'}^*) - (E_v - E_{v'})\right| > \delta\epsilon, \tag{8}$$

must hold; $\delta\epsilon$ is the width of a vibrational level. Pigment molecules in solution at room temperature display little or no vibrational structure in either absorption or emission spectra. Thus, $\delta\epsilon$ is likely to be so large that condition (8) cannot generally be fulfilled.

(c) *Formation of "localized" excitons:* Davydov[9] treats "localized" excitons in molecular crystals. These are associated with local lattice deformations which travel together with the excitation[10] and generally shorten the excitation lifetime. No similar effect appears to have been described in liquid solutions.

Distortions in a molecular crystal are caused by resonance interactions between like molecules. Since there are usually no resonance interactions between pigment and solvent molecules, the formation of "localized" excitons is unlikely in a solution. If present, "localized" excitons should shorten the excitation lifetime. Hence, "localized" excitons are restricted to quenching solvents and cannot be used to argue for localization in weak interactions in general.

3. *Ensemble Averages in the Incoherent Limit.*—As noted earlier, coherence among the amplitudes, a_k, of the localized states, ϕ_k, is interrupted by collisions in the weak-interaction case. In this case the energy transfer involves broad spectra, and in the incoherent limit the perturbation problem may be treated by Fermi's "Golden Rule."[11] Equations (1) and (2) may then be replaced by

$$(d/dt)|a_i|^2 = \sum_k F_{ki}(|a_k|^2 - |a_i|^2), \tag{9}$$

where the F_{ki} are pairwise transfer rates,[12] and we have neglected radiation. Since, in general, all of the $|a_i|^2$ are nonzero, (9) also corresponds to a delocalized picture.

One may also consider an ensemble of systems in each member of which (at any

instant of time) one of the $|a_i|^2 = 1$, and all the others are zero. If the relative number of systems in which $|a_j|^2 = 1$ is given by the solution of (9) for $|a_j|^2$, then the ensemble of localized-excitation systems has the same average properties as an ensemble of (identical) delocalized-excitation systems. If one follows the time course of a single member of the ensemble, the energy appears to "jump" around as a particle in Brownian motion. The localized ensemble leads to the random walk problem of mathematics. It has been known for some time[13] that the random walk problem may also be solved by differential equations of the form (9).

If the density of (dye) molecules in the aggregate is high, it is possible to approximate the system of differential equations (9) by a diffusion equation. Such a calculation is applied to the photosynthetic unit in a subsequent paper.[14]

In regard to the use of the localized picture, some errors occurred in the past. If the number of "jumps" to a specific site (considered to be a trap) is known from the solution of the random walk problem, it is incorrect to calculate the trapping time as the product of that number and the pairwise transfer time. If a molecule has several nearest-neighbors, the "jump" into one of them occurs more rapidly because of the others. (A small additional diminishing of the mean "jump" time is produced by the effect of the nonnearest neighbors.) Thus, for example, if two weakly interacting molecules are isolated in solution with separation r and (pairwise) transfer time t_1, then in a cubic lattice of constant r of the same molecules, the actual transfer time is approximately $0.12\ t_1$. The delocalized mathematical treatment takes account of the branchings (many neighbors) automatically.

The delocalized description has a further advantage for ensembles of systems in each of which the probability of initial excitation is equal for all molecules. The differential-equation system of such an ensemble can be solved using the uniform delocalization of the excitation energy as the initial condition.

The localized and delocalized pictures lead to the same ensemble averages in the incoherent limit. On the other hand, it must be emphasized that complete incoherence can never be achieved in the presence of resonance interactions, however weak.[15] Thus, the equations (9) are not completely valid, and hence the delocalized picture (with the possibility of relocalizations) is the only tenable one.

Summary.—(1) In excitation transfer, energy must be considered delocalized unless the presence of specific localizing processes is demonstrated. (2) A criterion for localizing processes is given. On the basis of this criterion, it is found that, for weak interactions, localizing processes in liquid solutions at room temperature are unlikely. (3) Localized and delocalized pictures give equivalent ensemble averages in the incoherent limit. Since this limit cannot be reached in the presence of resonance interactions, the equivalence is only approximate, and only the delocalized picture is generally valid.

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[15] Complete incoherence can only be achieved with infinitely high collision rates, which would lead to infinitely broad spectra.

STUDIES ON CYCLOSERINE-CATALYZED REACTIONS

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The reaction of diisopropylphosphofluoridate (DFP) with chymotrypsin and other serine esterases has been well documented in literature[1-4] and the sequence of amino acids of the peptides containing the diisopropyl phosphoryl (DIP) moiety from various DFP-inhibited serine esterases has been established.[5-10] The phosphoryl moiety appears to be located on a serine residue in the enzyme protein. Also, the specific acetylation of chymotrypsin with p-nitrophenyl acetate originally reported by Balls and Aldrich[11] has been shown to result in the acetylation of the serine hydroxyl group which presumably is the same serine residue as that involved in the phosphorylation with DFP.[12] Several mechanisms have been proposed to explain the catalytic action of chymotrypsin and other esterases susceptible to inhibition by DFP.[13-16] However, none of the model systems proposed, e.g. the oxazoline model[15] and the bicyclic intermediate formed from aspartyl serine peptide,[16] was either catalytic or able to react with DFP.

During an investigation on the second phosphorylation site in DIP-trypsin,[17] a new amino acid was observed and on the basis of its chromatographic and electrophoretic behavior and its susceptibility to periodate oxidation, it was tentatively concluded to be hydroxylysine.[18] However, in the course of the isolation of this new amino acid on a large scale, it was found to differ from δ-hydroxylysine in its chemical properties despite its striking resemblance to the latter on the basis of criteria earlier employed. Some properties[18a] of this new amino acid suggested that it could be a derivative of hydroxylamine. Several hydroxylamino acids, ε-N-hydroxylysine[19] and δ-N-hydroxyornithine,[20, 21] and hydroxylamine derivatives of amino acids, cycloserine,[22, 23] have been reported in recent years. A study of the properties of some of these hydroxylamino acids was undertaken with the idea of

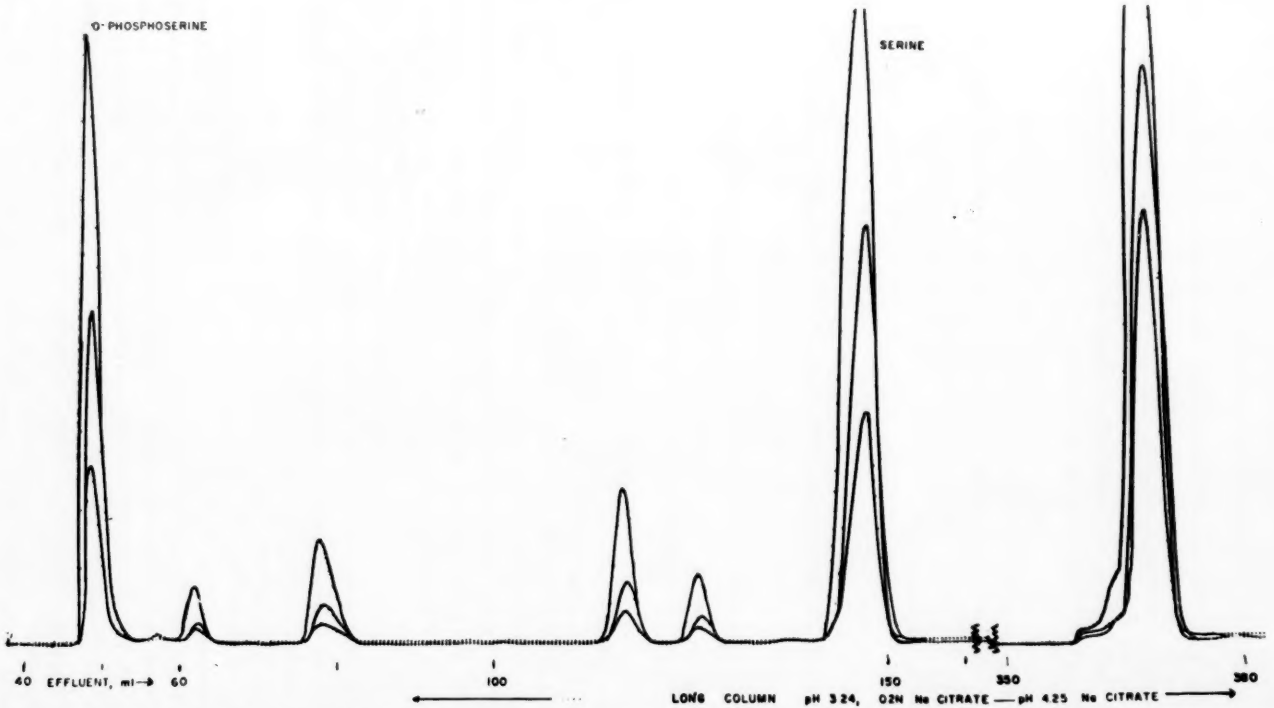


FIG. 1.—Chromatogram of the partial acid hydrolysate of the DFP-treated cycloserine (see text for details). (The original chromatogram has been condensed here. With the exception of a very small peak at 228 ml effluent volume, no other ninhydrin-positive components were observed in the portions not shown by this figure.)

obtaining information potentially pertinent to the isolation and characterization of the unknown amino acid observed in trypsin and chymotrypsin.

This paper deals with the catalytic properties of one such hydroxylamino acid, cycloserine.[22] The studies reported herein were prompted by the observation that cycloserine could catalyze the hydrolyses of DFP (which is also catalyzed by imidazole and hydroxylamine) and of p-nitrophenyl diethyl phosphate (E600) (hydroxylamine being the only other known model compound that can function in a similar manner with this latter compound).

Materials.—Cycloserine was prepared from L-serine methyl ester according to the procedure described.[24] The material crystallized in the form of rosettes and was found to have two titrable groups, one with a pK of 4.10 and another with a pK of 7.10–7.20. Its purity was further established by the absence of serine or other amino acids. One of the preparations of cycloserine was carried out under more alkaline conditions than normally employed.[24] The high alkaline pH led to dimerization[23] of cycloserine to the diketopiperazine form, and the product isolated was a mixture of cycloserine and its diketopiperazine. This preparation was characterized by its relatively low solubility in water as compared to cycloserine and by the absence of stoichiometry between the group with pK 4.10 and that with a pK of 7.10. Titration studies suggested that about 75% of the material in the above-mentioned preparation was in the diketopiperazine form. DFP was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Cinnamoyl imidazole (CI) was obtained from Dr. S. A. Bernhard. Carboxypeptidase-A, subjected to treatment with DFP, was purchased from Worthington Biochemicals, Freehold, N.J.

Experimental.—Four hundred mg of cycloserine in 9 ml of water were treated with 400 mg of pure DFP in 1 ml of isopropanol. This solution, originally at pH 3.8, was brought to pH 6.80 by the addition of 0.5 *N* KOH and maintained at that pH with the aid of an automatic titrator.[25] The reaction was accompanied by the consumption of alkali. At the end of 10 hr, when the alkali consumption had considerably slowed down, the solution was adjusted to pH 6.0 and evaporated to dryness under reduced pressure. The residue was dissolved in water and desalted by passing over Dowex 50 (H^+) column, and the material eluted with dilute NH_4OH. The ammonium hydroxide eluate was taken to dryness, and the residue was dissolved in 15 ml of redistilled, constant boiling HCl and subjected to partial hydrolysis in a sealed tube for 90 min at 105°C. The hydrolysate was evaporated to dryness under reduced pressure, and traces of HCl were removed by repeated additions of water followed by evaporation to dryness. The hydrolyzed residue was made up to 50 ml with water. A small sample was analyzed for amino acids by the procedure of Spackman, Stein, and Moore[26] with the aid of a Beckman-Spinco model amino acid analyzer (results in Fig. 1). The chromatogram showed the presence of an acidic amino acid, serine, and another component emerging at 370 ml of effluent volume of the buffer.

The DFP-treated cycloserine (following incubation at pH 6.8) was also analyzed for any free amino acids that might be present by means of the amino acid analyzer. As with cycloserine, no ninhydrin-reacting component was observed when such analyses were made either on the long 150-cm column for acidic and neutral amino acids or on the short 15-cm column used for the determination of basic amino acids. However, exposure of the DFP-treated cycloserine to alkaline pH (9.5) for 10 min and subsequent analyses resulted in the formation of a ninhydrin-positive component that emerges at 29 ml of the effluent volume on the 15-cm short column. The nature of this amino acid has not been identified.

Results.—The acidic component present in the hydrolysate was isolated by a column chromatographic technique similar to the one used in the analytical procedure mentioned above and was desalted. It was identified as o-phosphoserine by its mobility on the amino acid analyzer col-

TABLE 1

DATA PERTAINING TO THE IDENTIFICATION OF PHOSPHOSERINE OBTAINED FROM THE PARTIAL ACID DIGEST OF DFP-TREATED CYCLOSERINE

No.	Time of hydrolysis (hr)	Phosphoserine (μmoles)	Serine (μmoles)
1	0	1.39*	0.047
2	2	0.36	0.77
3	10	0.032	1.14
4	12	0.032	0.96

* Phosphate analysis revealed 2 μmoles of in organic phosphate in the H_2SO_4 digest of the sample.

TABLE 2

THE FIRST-ORDER RATE CONSTANTS OF CI HYDROLYSIS IN THE PRESENCE OF CYCLOSERINE

pH*	First-Order Rate Constants (moles/sec.)* Cycloserine†	Cycloserine-diketopiperazine mixture‡
4.35		3.0×10^{-3}
4.85		2.59×10^{-3}
5.30	1.87×10^{-3} (2760)§	1.67×10^{-3}
5.98	2.86×10^{-3} (2770)	1.06×10^{-3}
6.86	1.8×10^{-3} (2810)	7.8×10^{-4}
7.85	1.2×10^{-3} (2750)	
8.90	2.4×10^{-4} (2730)	
9.18		7.1×10^{-4}

* All values reported here were obtained after correction for the spontaneous hydrolysis of CI.

0.025 *M* acetate buffer between pH 4.35 and 5.30; 0.025 *M* phosphate buffer between pH 6.0 and 8.0; and 0.025 *M* pyrophosphate buffer at pH 9.0.

† Cycloserine concentration 1.67×10^{-3} *M*; CI concentration 2×10^{-4} *M* in a final volume of 3.0 ml.

‡ Cycloserine concentration 1.33×10^{-3} *M*; CI concentration 6.7×10^{-5} *M* in a final volume of 3.0 ml.

§ The absorption maximum of the peaks at very low and high pH were shifted toward shorter wavelengths presumably due to the presence of cinnamate ion (absorption maximum 2690 Å) arising from the spontaneous hydrolysis under these conditions.

The values shown in the parentheses indicate the wavelength of maximum absorption at the end of the reaction.

umn, by its conversion to serine on prolonged acid hydrolysis, and by the determination of its phosphorus content.[27] The results obtained in these studies are shown in Table 1. Nearly 200 μmoles of o-phosphoserine were isolated in the experiment described above.

Treatment of the above-mentioned cycloserine-diketopiperazine mixture with DFP, followed by analyses of the partial hydrolysate under conditions identical to those used earlier, showed several acidic ninhydrin-positive components in addition to o-phosphoserine (Fig. 2). Exposure of this partial acid hydrolysate to the action of carboxypeptidase at pH 7.0 for 2 hr resulted in the disappearance of the components represented by peaks II and III and a simultaneous increase in o-phosphoserine and serine. These results indicate the presence of phosphoryl derivatives of serylserine dipeptides in the partial acid hydrolysate arising from the diketopiperazine.

Reaction of cycloserine with cinnamoylimidazole: The elegant use of cinnamoylimidazole for studying the acylation and deacylation mechanism of chymotrypsin-catalyzed reactions has been reported by Bender, Schonbaum, and Zerner.[28] The influence of cycloserine on the hydrolysis of CI was investigated, and the results of a typical experiment are shown in Figure 3. The catalytic action of cycloserine was pH-dependent. The apparent first-order rate constants, obtained under conditions of excess of cycloserine at various pH's are given in Table 2. The reaction was accompanied by the disappearance of the absorption peak at 3,070 Å. At longer time this 2,810 Å peak decayed appreciably, the wavelength of maximum absorption shifting toward shorter wavelengths presumably due to the appearance of cinnamate ion (absorption maximum 2,690 Å). The reaction forming the intermediate with λ_{max} = 2,810 Å is primarily due to the acylation of cycloserine. The deacylation of this acylated product to cycloserine and cinnamate ion is a comparatively slow process.

Deacylation of the intermediate formed by the reaction of CI with cycloserine: The deacylation rate of the cinnamoyl cycloserine product was followed in the pH-stat at pH 7.0 and 9.0. The process followed first-order kinetics. The half times for deacylation at pH 7.0 and 9.0 were of the order of 24 hr and 7 hr, respectively. Cinnamate ion and cycloserine were identified during the deacylation process. Thus, the action of cycloserine appears to be catalytic, the rate-limiting step in the process being the deacylation of the intermediate. This was further confirmed by the fact that cycloserine could catalyze the hydrolysis of more than one equivalent of CI. The rate of deacylation of the intermediate was unaffected by the addition of imidazole. The pH dependence of the catalyzed hydrolysis of CI in the pres-

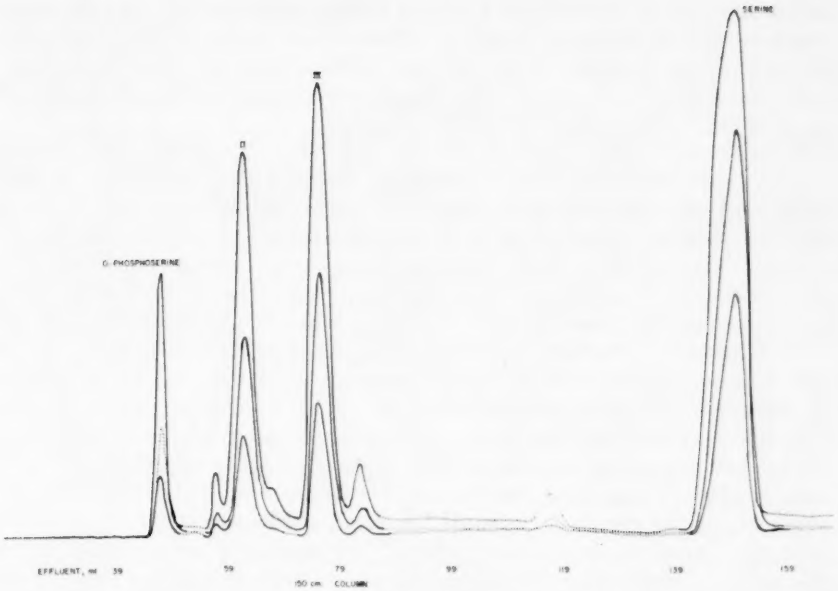


FIG. 2.—Chromatogram of the partial acid hydrolysate of DFP-treated cycloserine-diketopiperazine mixture (see text for details). Partial hydrolysis was achieved by refluxing the reaction product with constant boiling HCl for 90 min.

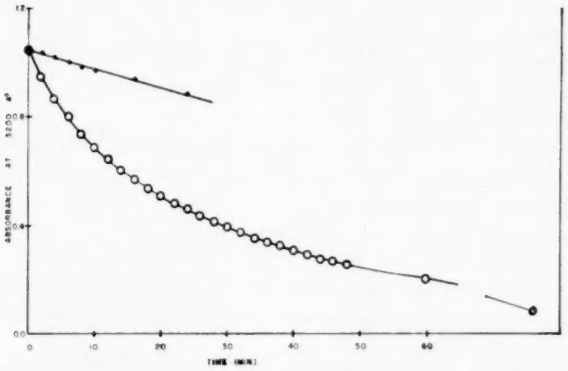


FIG. 3.—Cycloserine-catalyzed hydrolysis of CI. Cycloserine, 1.67×10^{-3} *M*; CI, 2×10^{-4} *M* in 3.0 ml of 0.025 *M* phosphate buffer, pH 6.86.

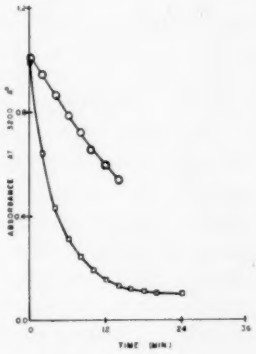


FIG. 4.—Cycloserine-diketopiperazine mixture catalyzed hydrolysis of CI. Cycloserine, 1.33×10^{-2} *M*; CI, 6.7×10^{-5} *M* in 3 ml of 0.025 *M* acetate buffer, pH 4.35.

ence of the cycloserine-diketopiperazine mixture was also investigated. Figure 4 illustrates the catalysis observed with such a preparation at pH 4.35. The first-order rate constants calculated for this mixture plus cinnamoylimidazole are summarized in Table 2. The intermediate formed by the acylation of cycloserine

during the reaction with CI fails to give a positive hydroxamate test upon direct treatment with acidic ferric chloride.[29] However, treatment of the intermediate with 0.2 *M* NH_2OH at pH 5.5 for 5 min and subsequent treatment with acidic ferric chloride yields the cinnamoyl hydroxamate-ferric chloride complex (characterized by its strong visible (purple) absorption spectrum).

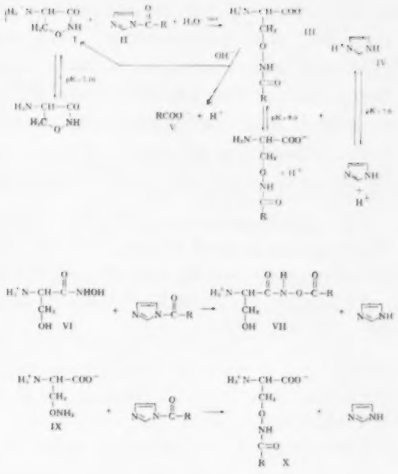
Protonic equilibrium associated with the cycloserine-CI reaction: The reaction between cycloserine and CI was studied in the pH-stat to follow the protonic equilibrium accompanying the process. Five mg of cycloserine in 6 ml of water at pH 7.0 and 10 mg of CI in 2 ml of acetonitrile were mixed, and the reaction was followed in the pH-stat. Under these conditions, no pH change was observed for nearly 120 min. There was neither release nor uptake of protons. A study of the absorption spectra at various intervals during this 2-hr period revealed that the 3,070 Å absorption maximum was steadily diminishing with the concomitant formation of an intermediate with absorption maximum at 2,810 Å. At the end of 2 hr, the absorption at 2,810 Å was nearly constant. However, over longer time intervals a steady uptake of alkali was noted. This latter process was followed for 10 hr at pH 7.0, and then the pH was raised to 9.0. The alkali uptake at pH 9.0 was faster than at pH 7.0, suggesting that the deacylation step is OH^- concentration-dependent. At the end of 35 hr at pH 9.0, alkali uptake was complete, indicating the completion of the deacylation reaction. The amount of base consumed during this period corresponded to the amount of cinnamate to be expected from the deacylation process. The final product formed was identified as cinnamic acid by isolation from the acidified reaction mixture and characterization by ultraviolet spectra (in 0.050 *M* phosphate buffer, pH 6.86, absorption maximum 2,695 Å) and by the determination of its pK in 60 per cent dioxane-water mixture (pK observed 6.2). Cycloserine was also recovered after the completion of the reaction.

When cycloserine was treated with CI at pH 5.0 under conditions similar to those used at pH 7.0, the formation of the acyl intermediate was accompanied by the uptake of one mole of proton per mole of the reactants.

Discussion.—The isolation of o-phosphoserine from the partial acid hydrolysate of the DFP-treated cycloserine attests to the phosphorylation of the serine hydroxyl group. Model serine peptides[16] and oxazolines[15] do not react with DFP under similar conditions. O-phosphoserine was also identified in the partial acid hydrolysate of cycloserine treated with DFP at pH 5.0. This observation, as well as the observed presence of o-phosphoserine in the hydrolysates of cycloserine-diketopiperazine mixture (see Fig. 2), suggest the ring nitrogen in the cycloserine molecule to be the initial site of phosphorylation.

The reaction between cycloserine and CI is characterized by the rapid formation of an acyl intermediate; the next step, i.e., the deacylation process which is relatively slow, limits the catalytic power of cycloserine. The deacylation reaction has been demonstrated by the following: (*a*) Cinnamic acid was identified as the product of the reaction. (*b*) Cycloserine could be recovered almost quantitatively at the end of the reaction. (*c*) Cycloserine could hydrolyze more than one equivalent of CI via the two-step sequence described above. The protonic equilibrium accompanying the reaction offers valuable information concerning the nature of the intermediate formed. Assuming the proposed structure for cycloserine[22] to be valid, the scheme of events given below satisfactorily explains the data obtained.

The formation of the intermediate III with the release of imidazole at pH 7.0 would satisfactorily account for the lack of proton uptake at pH 7.0 and the consumption of one equivalent of protons at pH 5.0. Other mechanisms involving (*a*) seryl-N-hydroxamate, VI, leading to the formation of seryl N,O-diacyl hydroxamate intermediate, VII,[30] and (*b*) o-seryl hydroxylamine, IX, yielding the intermediate X appear to be excluded by the results of protonic equilibrium studies. It is pertinent to emphasize that the scheme proposed here is merely a general representation of the over-all reaction, and no attempt will be made at this time to present the actual mechanism involved.



A comparative study of the reactions of cycloserine and the proteolytic enzyme chymotrypsin reveals some striking similarities. Both chymotrypsin and cycloserine are inhibited by DFP, and acid hydrolysis of their DIP-derivatives yields o-phosphoserine. Likewise, the acyl intermediates seem to have similar absorption maxima and give positive hydroxamate tests with acidic ferric chloride upon treatment with NH_2OH at pH 5.5, unlike esters such as o-acyl serine peptides which give negative hydroxamate tests under these conditions.

As much as these similarities between cycloserine- and chymotrypsin-catalyzed reactions provide a basis for the speculation as to the nature of the active serine in chymotrypsin and other serine esterases, the need for definite and conclusive evidence in this regard must be recognized. Since the α-carboxyl group of the serine would be in peptide linkage with the amino group of the adjacent amino acid residue in the peptide chain (e.g., with the NH_2 group of a glycine residue in the reported sequence of phosphopeptides isolated from serine esterases), the chances of cycloserine *per se* being present in the enzyme proteins are indeed remote. However, the possibility that the $-ONH_2$ group of cycloserine (if present in the enzyme proteins) coupled with a β-COOH of an aspartic acid residue adjacent or in close proximity to it, i.e., a linkage similar to that present in cycloserine with a hydroxylamine bridging a carboxyl and a serine hydroxyl, should not be overlooked. The extreme reactivity of the model peptide containing aspartic acid, serine, and hydroxylamine,[31] and of serine esterases may well be due to such linkages resulting in the formation of structures with unusual catalytic properties.

Furthermore, the enhanced catalytic activity of the cycloserine-diketopiperazine mixture at low pH (see Fig. 4) implies that the $-ONH_3^+$ group of the o-serylhydroxylamine could be associated with catalysis in some instances. It should be emphasized, however, that the ultimate proof for the unique reactivity of a single serine residue in chymotrypsin and other esterases must await further structural studies designed to elucidate the factors that confer such properties on the serine.

Summary.—The reaction of cycloserine with DFP and cinnamoylimidazole was

investigated. O-phosphoserine was isolated from the partial acid hydrolysate of the DFP-treated cycloserine. The reaction between cycloserine and cinnamoylimidazole was characterized by a rapid formation of an acyl intermediate; the deacylation of this intermediate was found to be slow. The acyl intermediate gave a positive hydroxamate test with acidic ferric chloride only upon treatment with hydroxylamine at pH 5.50. The implications of the many similarities between the chymotrypsin- and the cycloserine-catalyzed reactions are discussed.

The author wishes to thank S. A. Bernhard, Y. Shalitin, J. H. Carter, and L. A. Cohen (National Institutes of Health, Bethesda, Md.) for their helpful discussions. These investigations were supported by a grant from the National Science Foundation to Dr. Bernhard.

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THE ORIGIN OF SPONTANEOUS MUTATIONS DURING MEIOSIS

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The spontaneous mutation rate (reversions per nucleus per division) of three independent mutations tested in cells of *Saccharomyces cerevisiae* undergoing meiosis is 6–20 times that of cells mitotically reproducing (Magni and von Borstel[1]).

Operationally, the mechanisms responsible for the increase in mutation rate at meiosis can be grouped into two general categories: (1) biochemical changes occurring in the cell during the meiotic process, such as a possible reduction of enzymatic activities necessary for the repair of premutational damages (evidence for depression of other enzymes is available, for instance, those involved in respiration according to Ogur's personal communication, and in galactose fermentation in my own unpublished observations) or other possible changes in the chemical components indispensable for the structural differences between a vegetative and a sporulating cell; and (2) differences in chromosome behavior (pairing and crossing-over) between mitosis and meiosis.

Discrimination between these is possible by genetic analysis.

Materials and Methods.—(a) *Symbols:* a and α. mating type genes; MA_2, capacity of fermenting maltose; thr, threonine dependence; hi, histidine dependence; ar, arginine dependence; is, isoleucine dependence; tr, tryptophane dependence; met, methionine dependence; ad, adenine dependence.

(b) *Strains:* All strains used have been prepared by introducing the proper markers through conventional crosses and genetic segregation. The mutant $thr_{4\text{-}1}$ and the deletion at the thr_4 locus in chromosome III was kindly supplied by Dr. D. C. Hawthorne; the mutants thr_2, $hi_{1\text{-}1}$, ar_6, is_1, tr_2, by Dr. R. K. Mortimer; met_3, by Dr. C. C. Lindegren.

(c) *General procedures:* For culture media, removal of the ascus wall, and methods for estimating spontaneous mutation rates during vegetative and meiotic division cycle, see ref. 1.

(d) *Identification of external suppressors in the revertants:* It has been necessary in this investigation to verify whether prototrophic revertants derived from auxotrophs are due to external suppressors or to intracistronic events (true back-mutations or intracistronic suppressors). For the present purposes the distinction between the two intracistronic events is not relevant.

Every haploid revertant (either derived from haploid spores or segregated from diploid vegetative cells) was crossed to a haploid strain of the opposite mating type carrying an ad_2 mutant which produces a red pigment in haploid colonies or in homozygous diploids.

The diploid obtained from the cross was sporulated and the ascus wall lysed by means of BPE extract (for details, see ref. 1). After 2 min of sonication for dissolving spore clumps, the suspension was seeded on complete medium and allowed to grow for 3 days. All the colonies were replicated on a medium (I) with the amino acid, and on a medium (II) without the amino acid required by the ancestor of the revertant.

Only the colonies which were pink on the complete medium were scored because their color indicated that they were haploid and therefore originated from a single spore. When a revertant is produced by an external suppressor, auxotrophs will segregate which do not grow on medium (II) while growing normally on medium (I). The proportion of such segregants among the haploid pink colonies gives an estimate of the linkage between the original mutant gene and its suppressor. For every revertant, 500–600 (in few cases around 1000) haploid segregants were analyzed. When such a test is completely negative (no auxotrophs segregating out of 500 haploids), it can be concluded that no external suppressors are present at a distance greater than 0.2 unit from the mutant gene.

Results.—Effect of a chromosomal deletion on meiotic mutations: For testing the

possible contribution of biochemical changes to the increase of meiotic mutation rate, we designed an experiment with a yeast strain in which pairing and chromosomal exchanges were rendered impossible at the specific locus analyzed for reversion.

As demonstrated by Hawthorne,[2] the behavior of the aberration in chromosome III which includes the thr_4 locus can be summarized as follows: (1) it is lethal in the haploids and segregates 2:2 in the asci from an heterozygote; (2) it is closely linked to the *a* mating type, and extends to within 5 units of the MA_2 gene which is normally about 40 units distal from *a*; (3) the original heterozygous stock had no history of the thr_4 mutant in its pedigree and, when spores carrying the lethal marker are crossed to α thr_4 haploids, all the resulting hybrids are threonine dependent. The aberration, therefore, meets the three genetic requirements for a long chromosomal deletion: lethality in haploids, reduction of crossingover, and a pseudo-dominance effect.

Making use of the thr_4 deletion, we constructed and tested the strains shown in Table 1 for reversion to threonine independence.

TABLE 1

MUTATION RATES ($\times 10^{10}$)

(Reversions per cell per generation from threonine dependence to independence)

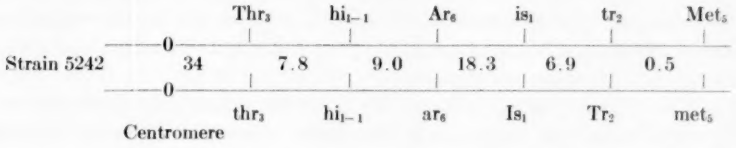
Strain		Mitosis	Meiosis
5243	α thr_{4-1} MA_2 / 0 / 0 / a thr_{4-1} MA_2	3.23 ± 0.16	99.2 ± 8.5
5244	α thr_{4-1} MA_2 / 0 / 0 / a MA_2	2.67 ± 0.19	3.80 ± 0.42
10267	a thr_{4-1} MA_2 / 0	1.91 ± 0.10	...
10268	α thr_{4-1} MA_2 / 0	1.20 ± 0.13	...

It can be seen that strain 5243, diploid homozygous for thr_{4-1}, shows, as expected, a mitotic mutation rate to independence twice as high as the average between the two haploids. Strain 5244, heterozygous for the deletion, appears, on the other hand, to revert mitotically at a rate 60 per cent higher than expected. Fluctuation among strains are, of course, not surprising, since a new genetic background has been introduced when the deletion stock was constructed.

The major finding in this experiment is the difference between mutation rates at meiosis in the two diploid strains. The one heterozygous for the deletion does not show any significant increase in mutation rate, while the normal homozygous strain 5243 produces approximately 30 times more revertants per meiotic division. Since the meiotic process proceeds quite normally in both strains, it can be concluded that the *meiotic behavior of chromosomes and not biochemical changes is responsible for the increased spontaneous mutation rate during meiosis.*

A more decisive experiment could be performed with a strain carrying the heterozygous thr_4-deletion and another marker in a homozygous state. This would allow the analysis of reversion rates for threonine and the other biochemical requirement that occur during the same meiotic process. Unfortunately, our efforts to construct such strains have so far failed. Homozygosis for biochemical markers tends to reduce the frequency of sporulation in yeast. In the present case the introduction of any homozygous mutant into the strain containing the heterozygous thr_4-deletion led consistently to clones producing less than 2–3 per cent spores. Such a low frequency of sporulation makes any test for mutation technically impossible with the procedures presently used.

Mutations and chromosomal exchanges during meiosis: A diploid strain was constructed with the following fifth chromosomes:



The map distances shown here are the average of all the experiments (tetrad analysis and random spore analysis) performed with strain 5242 in the last two years. They are in good agreement with Hawthorne and Mortimer's[3] data, except for the region $thr_3 - ar_6$, where the distances were: $thr_3 - hi_1 = 1.25$, and except $hi_1 - ar_6 \cong 0.5$ (Hawthorne and Mortimer, personal communication). It is commonly found that crossingover frequencies vary often to a great extent when the genetic background is changed. It should be noted, however, that in our strain only the region $thr_3 - ar_6$ seems to be expanded by a factor of about 8, while the adjacent regions in the same chromosome remain the same. The explanation for this difference remains obscure.

In two independent large-scale experiments that gave an average mitotic mutation rate of 6.58×10^{-10} for hi_{1-1}, with a corresponding meiotic one of 1.8×10^{-8}, revertant and nonrevertant spores were isolated and their phenotypes analyzed. The frequencies of parental and recombinant classes for region $thr_3 - ar_6$ are given in Table 2.

TABLE 2

RANDOM SPORE ANALYSIS FROM STRAIN 5242

	Nonrevertant spores Phenotypes				Revertant spores Phenotypes			
	thr_3	hi_1	ar_6	N	thr_3	hi_1	ar_6	N
Parental	+	−	+	92	+	+	+	26
	−	−	−	80	−	+	−	21
Recombinants	+	−	−	17	+	+	−	71
	−	−	+	13	−	+	+	45
	Recombination = 0.15				Recombination = 0.71			

The association of reversion with chromosomal exchange is striking. Furthermore, the estimate given is a minimal one because, among the revertant spores, the crossingover frequency in the region $ar_6 - is_1$ was increased in our experiments from 36 per cent in the control to 51 per cent, showing that there is still a certain

amount of negative interference even far from the region considered. This is an indication that within that region negative interference is probably very strong. In consequence, the real recombination frequency between thr_3 and ar_6 among the revertant spores could be as high as the order of 0.90 and nearly equal to the percentage of revertants produced during meiosis.

The discrepancy between the two complementary classes of recombinants among the revertant spores (71 and 45) is at the limits of significance and could be due to two phenomena: partially reduced viability of threonine dependent spores, as shown also by nonrevertant spores, and possibly to a certain degree of polarization of the chromosomal exchanges connected with the mutational process.

Some of the revertants taken at random were examined for the presence of external suppressors by the procedure described in *Materials and Methods*. In 61 revertants no suppressors were found within 0.2 units of the hi_1 locus. Therefore, it can be concluded that *spontaneous reversions of* hi_{1-1} *mutant during meiosis can be attributed to intragenic events and are in the great majority or possibly in all instances associated with a chromosomal exchange.*

Mutations occurring during mitosis: In order to obtain data for a correct interpretation of the above findings, a genetic analysis was carried out on revertants at the hi_1 locus arising during mitotic division in haploid and diploid cells. From haploid strain 10102, carrying the hi_{1-1} mutation, 73 revertants which occurred during mitosis were isolated. From diploid strain 5242, homozygous for hi_{1-1}, 77 revertants were obtained. All revertants were independent of each other, since only one prototrophic clone was taken from each tube of growth inoculated with few cells (10^3–10^4).

The prototrophs derived from strain 10102 were tested for external suppressors. None were found within the limits of the sensitivity of the test.

The diploid revertants were sporulated, and at least six haploid clones derived from each one were genetically analyzed. The results obtained may be summarized as follows: (*a*) All diploid prototrophs were heterozygous (Hi_1/hi_1), proving that, without any exception, all the mutations were dominant. (*b*) Out of 77 mutations, 44 occurred in the chromosome Thr_3–hi_1–Ar_6–is_1–tr_2–Met_5, and 33 in the chromosome thr_3–hi_1-ar_6–Is_1–Tr_2–met_5. The difference between these figures is not significant, clearly showing that no preferential mutation occurred in either of the two chromosomes. (*c*) The 77 mutations were scored for external suppressors with 76 negative cases and 1 doubtful. This exception was a mutant which in our test gave one auxotrophic recombinant out of 1150 segregants analyzed. Therefore, the mutation could be attributed to a suppressor which showed a recombination frequency with the original site of the order of $8\text{–}9 \times 10^{-4}$. This frequency would appear to be within the limits of intragenic recombination and therefore the particular suppressor involved is probably an intracistronic suppressor.

A large scale experiment was set up for estimating the frequency of somatic crossingover in the region thr_3–ar_6 among revertant and nonrevertant cells in a vegetatively growing culture. Two hundred and seventy-eight independent mitotic revertants were individually studied by means of tetrad analysis, and a large number of control colonies were checked by replica plating. The results are summarized in Table 3.

TABLE 3

CORRELATION BETWEEN SOMATIC RECOMBINATION AND REVERSION IN STRAIN 5242 FOR THE hi_1 LOCUS

Type of cells	Total number	Recombinants in region thr_3-ar_8	Frequency of somatic crossingover	Upper and lower limits P = 0.5%*
Nonrevertants	28,631	2	1.4×10^{-5}†	4.39×10^{-4} 2.34×10^{-5}
Revertants	278	3	1.02×10^{-2}	3.84×10^{-2} 1.22×10^{-3}

* Estimated according to Fisher and Yates,[4] p. 48.
† The observed frequency is doubled because only one crossover out of two is expressed with homozygous genetic markers.

It appears entirely clear that most spontaneous mutants arising during a mitotic process in diploid cells are not associated with a chromosomal recombination. On the other hand, the frequency of somatic crossingover involving the region of the locus under investigation is about 700 times higher in the mutants than in the control cells. This means that when selection for revertants is practiced, it ensures selection for the rare exchange in the region.

We have often observed that the mitotic mutation rate in homozygous diploids is always a little higher than the double of the same rates in haploids (e.g., Table 4 in ref. 1). The difference was never significant but the trend was consistent. This fact can be best explained by assuming that, in addition to mutations of unknown origin, which ought to occur in haploids and diploids in a ratio of 1:2, there are mutations associated with chromosomal exchange and therefore restricted to diploid cells.

Discussion.—The major facts resulting from our previous investigation[1] and the present one are:

(*a*) In *Saccharomyces cerevisiae*, diploid strains homozygous for certain biochemical mutants (met_2, ar_8, hi_{1-1}, thr_{4-1}) show higher reversion rates in meiosis that in mitosis.

(*b*) The great majority of revertants occurring during a meiotic process are associated with chromosomal exchanges in the region of the specific locus.

(*c*) All revertants analyzed are the result of intragenic events and are not attributable to external suppressors. This fact is relevant, since it is known (Hawthorne and Mortimer[4]) that in *S. cerevisiae* external suppressors play an important role in the reversion from auxotrophy to prototrophy. Mortimer (personal communication) has also calculated that about 25 per cent of randomly chosen mutants are reverted more or less efficiently by supersuppressors.

(*d*) The great majority of revertants occurring during mitosis are not associated with crossingover phenomena, but the frequency of chromosomal exchanges in the specific region is highly increased among the revertants when compared with the nonrevertant cells.

These facts suggest that spontaneous mutations in meiosis arise as a consequence of unequal crossingover in a pairing-exchange-mutation sequence (cf. Lerman,[6] Magni[7]).

Let us assume that the original mutation was due to a loss or an addition of one or several nucleotides (not a complete triplet in any case) leading to a shift of the reading frame, and consequently to a nonsense protein or to the complete lack of a protein (Crick *et al.*[8]). An unequal exchange between two identically damaged

chromatids can restore a functional or partially functional sequence which will pass the restored instructions for a wild-type or a pseudo-wild-type protein.

Demerec[9] has postulated the same kind of mechanism of unequal crossingover to account for the increased reversion rate of arg A-162 mutant in *Salmonella typhimurium* when transduced by a phage carrying the same mutant in comparison with control, nontransduced bacteria. But more recently, Demerec[10] favored the hypothesis of increased mutability of the gene in the presence of a transducing fragment, because the mutation frequencies were increased, even when the donors were carrying a deletion covering the arg A-162 region. These last findings are at variance with our thr_4-deletion experiments.

It is likely that an unequal crossingover leading to a base loss or addition could revert a mutant of the "acridine type" (loss or addition, Brenner *et al.*[11]; Crick *et al.*[8]) more easily than would a mutant of the "base analogue" type (base substitution, Freese[12]).

The mutants which we have so far tested were chosen because they showed a low reversion rate and were not leaky; this property has been considered typical of the acridine type mutants.[8] Furthermore, no single external suppressor was found among our revertants. It is conceivable that external suppressors and particularly supersuppressors should be more effective on base analogue mutants than on acridine ones. Our mutations are all UV induced, and therefore at the present time there is no basis for presuming the molecular nature of the DNA damage involved.

Summary.—It is shown that it is the meiotic behavior of chromosomes and not biochemical changes that is responsible for the increased spontaneous mutation rate during meiosis. Spontaneous reversions of the hi_{1}-$_{1}$ mutant occurring during a meiotic process are, in the great majority, associated with a chromosomal exchange. This association suggests that spontaneous mutations in meiosis are caused by unequal crossingover.

The author is indebted to Drs. D. C. Hawthorne and R. K. Mortimer for supplying auxotrophic mutants, to Drs. R. K. Mortimer and R. C. Von Borstel for most stimulating discussions, and to Mr. Silvio Sora for his invaluable technical assistance.

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*THE ABSOLUTE STEREOCHEMICAL COURSE OF CITRIC ACID BIOSYNTHESIS**

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In 1948 Ogston noted that when such symmetrical molecules as citric (or aminomalonic) acid are acted upon by asymmetric enzyme surfaces, pairs of identical groups may well be differentiated.[1] Shortly thereafter it was shown, by using labeled substrates, that the two $-CH_2COOH$ groups of citric acid could, in fact, be distinguished.[2] When citrate is formed from oxaloacetate and acetyl-CoA by citrate synthase (E.C.4.1.3.7, condensing enzyme), and is then treated with aconitate hydratase (E.C.4.2.1.3, aconitase) to form *cis*-aconitate and *threo*-D_s-isocitrate, the $-CH_2COOH$ ends of these compounds are derived from acetyl-CoA, while the other atoms are derived either from oxaloacetate or water.[3-6] The acetyl-CoA-derived group yields acetate when citrate is cleaved by citrate-lyase (E.C.4.1.3.6, citratase),[7,8] and acetyl-CoA when citrate is attacked by the citrate cleavage enzyme.[9]

The nonequivalence of the two $-CH_2COOH$ groups is apparent when citric acid is represented as I (heavy line above, dotted lines below the plane of the paper). Carbon atoms 1 and 2 are to 5 and 4 as right arm is to left arm:[10] they cannot be superimposed except by reflection. The same molecule is shown in Emil Fischer projection as II (vertical lines below and horizontal lines above the plane of the paper). The carbon atoms are numbered according to the Hirschmann convention.[11] The major purpose of the present investigation has been to determine which $-CH_2COOH$ group, in the absolute stereochemical sense of I, is derived from acetate and which is derived from oxaloacetate.

In order to differentiate the two groups it was necessary to obtain asymmetrically labeled citric acid from a labeled compound of known absolute configuration. The following procedure is based upon the investigations, by Dangschat and H. O. L. Fischer, of the stereochemistry of quinic acid,[12] and by Davis and his associates of the biosynthesis of this compound.[13]

In the first stage quinic acid labeled with tritium in the 6 position was obtained with the aid of an extract of *Aerobacter aerogenes* A170-143 known to contain the enzymes quinate dehydrogenase (E.C.1.1.1.24) and 5-dehydroquinate dehydratase (E.C.4.2.1.10).[13] Thus 5-dehydroshikimic acid (III) yielded 5-dehydroquinic acid-6-T (IV), which on reduction gave quinic acid-6-T (V). The symbol $\sim$ indicates that the configuration at the carbon atom bearing tritium is unknown. In the second stage V was oxidized to citric acid-4-T (VII) by the method shown.[14] Lastly, the properties of (VII) as a substrate for aconitate hydratase were studied.

Materials and Methods.—Enzyme preparations: Cells of *A. aerogenes* A170-143 were grown, with stirring at 37° for 24 hr, on minimal medium A[15] supplemented with 20 mg of yeast extract (Difco) and 600 mg of quinic acid (volume 1 liter). Mucoid growth was not observed. The post log phase cells were washed with water, suspended in 0.033 *M* Tris-HCl[16] buffer, pH 7.5, and sonically disrupted. The initial extract (42 mg of protein from 1 ml of packed cells) showed strong NADH oxidase activity. After treatment with $MnCl_2$[13] and dialysis against 0.033 *M*

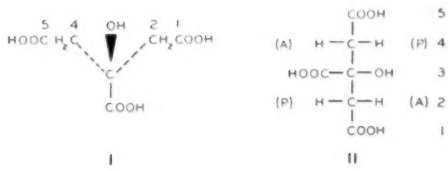

FIG. 1.

Tris-HCl buffer, pH 7.5, this activity was found to be negligible. The preparation (26 mg of protein) contained both quinate dehydrogenase and 5-dehydroquinate dehydratase. The specific activity of the former was 1.06 units/mg of protein at pH 9.4.[13] A small amount of 5-dehydroshikimic acid brought about the rapid oxidation of NADH (pH 7.4, 0.72 units/mg of protein). No appreciable losses of these activities occurred on storing the frozen solution at $-10°$ or on lyophylization. The *A. aerogenes* mutant was a gift of Dr. Bernard Davis.

Aconitate hydratase was prepared from pig heart and stored as a frozen solution.[17] Before use it was activated with Fe^{2+} and cysteine. Isocitrate dehydrogenase (E.C.1.1.1.42) and glutamate dehydrogenase (E.C.1.4.1.3) in phosphate buffer were purchased from Boehringer.

Throughout this paper a unit of enzyme is defined as that amount which catalyzes the transformation of 1 μmole of substrate/min at room temperature under optimum or specified conditions.

Quinic acid-6-T: (*a*) From 5-dehydroshikimic acid: To the lyophylized enzymes (0.43 mg protein), buffer pH 7.4 (Tris-HCl, 100 μmoles in 33 μl), 5-dehydroshikimic acid (8 μmoles), tritiated water (0.25 ml), and, finally, NADH (13 μmoles) were added and the reaction mixture left at 24°. The oxidation of NADH was followed by diluting 5 μl aliquots to 1 ml with 0.1 *M* NaOH (added to terminate the reaction) and observing the decrease in absorbancy at 340 mμ. The oxidation was complete in 35 min, at which time further reaction was prevented by adding HCl (100 μmoles). The tritiated water was removed by vacuum sublimation. Water was added and then removed by distillation, and this operation was repeated until all the easily exchangeable tritium had been displaced. The specific activity of the water in the reaction mixture was 578,000 cpm/μatom. The 5-dehydroshikimic acid was a gift of Dr. P. R. Srinivasan.

(*b*) From quinic acid: The enzymes (0.43 mg of lyophylized protein) in tritiated water (0.3 ml) were added to a lyophylized mixture of Tris-HCl buffer pH 7.4 (100 μmoles), NAD^+ (1 μmole), and potassium quinate (10 μmoles). The reaction mixture was maintained at 24° for 2 hr and 37° for a further 6 hr. Samples were withdrawn at various times, and the extent of incorporation was estimated. The specific activity of the water in the reaction mixture was 1×10^6 cpm/μatom. The reaction was terminated as in experiment (*a*).

Citric acid-4-T by oxidation of quinic acid-6-T: A sample containing less than 10 μmoles of tritiated quinic acid was chromatographed on a Dowex 1 acetate column.[18] Measurements of radioactivity served to locate the quinic acid peak. The combined peak fractions were concentrated to dryness, periodic acid (100 μmoles, 0.1 ml) was added, and the solution left in the dark at 24° for 1 hr. Under these conditions quinic acid consumes exactly two molar proportions of periodate, the reaction being complete in about 10 min. Periodic and iodic acids were removed with the aid of a Dowex 1-X8 formate column (7 × 0.7 cm). The column was developed with 3 *N* formic acid and the first 16 ml of effluent were concentrated to dryness. Water (2 ml) and bromine (10 μl) were added and the solution left in the dark at 27° overnight. The excess bromine was removed with a current of air and the reaction products were chromatographed on a Dowex-1 formate column.[18] The citric acid was located by radioactivity measurements. The over-all yield, based on radioactivity, was about 50%. The yield was not improved by adding strontium carbonate to the bromine oxidation mixture.[19] The specific activity of the citric acid from quinic acid was: method (*a*) 240,000 cpm/μmole, and (*b*) 435,000 cpm/μmole.

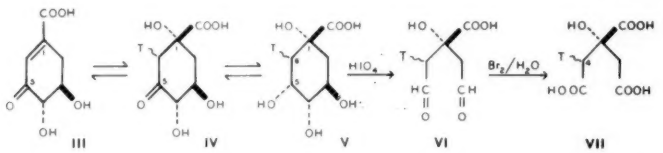

FIG. 2.

The oxidation process was studied in greater detail. A sample containing quinic acid (176,000 cpm) labeled by method (*b*) was diluted with potassium quinate (250 μmoles), and the solution chromatographed as above. The amount of acid in the various dried fractions was determined by titration, and 93% of the applied radioactivity was associated with the quinic acid peak. An isotope effect was observed: the specific activity fell linearly by 26% from the front to the tail of the peak; mean 617 cpm/μmole. The sodium quinate fractions were combined and the solution was passed through a short Dowex 50-H^+ column to give the free acid which was then oxidized. When the final reaction mixture was chromatographed, a series of peaks were recorded. The fourth and last peak corresponded to citric acid (33% yield). The specific activity of the acid increased by about 10% from the front to the tail of the peak. The mean specific activity (620 cpm/μmole) was in close agreement with that of the quinic acid.

Tritiated citric acids for control experiments: From isocitrate+T_2O: *threo*-D_s-isocitric acid (from crassulacean plants) was treated with aconitate hydratase in the presence of tritiated water and the enzyme destroyed by heating. The residual isocitrate was converted to α-ketoglutarate with isocitrate dehydrogenase, and the mixture of acids separated by silica gel chromatography.[20] Tritium was not incorporated into *cis*-aconitic acid. The incorporation into citric acid was 60% of the equilibrium value assuming labeling at a single position.[21] Specific activity: 85,000 cpm/μmole.

From isocitrate-3,4,4-T: A mixture of the four possible isocitric acids was prepared by the method of Fittig and Miller[22, 23] using tritiated succinic acid (New England Nuclear). The *threo*-D_sL_s mixture was separated from the *erythro*-D_sL_s mixture [(±)-alloisocitric acid] and from succinic acid by chromatography on a Dowex 1 formate column.[18] The *threo*-isocitric acids were eluted slightly ahead of the *erythro* mixture (ratio *threo*:*erythro*, 22:1). The *threo* mixture was treated with aconitate hydratase, and the citric acid formed was isolated by silica gel chromatography. Specific activity: 386,000 cpm/μmole.

From isocitrate-2-T: A mixture of the four stereoisomers of isocitric acid in roughly equal proportions (from New England Nuclear; synthesized by the method of Wislicenus and Nassauer[24]) was treated with aconitate hydratase, and the citric acid formed was isolated by silica gel and ion exchange chromatography. Specific activity: 50,000 cpm/μmole.

In each of these preparations the treatment with aconitate hydratase was continued for at least six times the period required to establish equilibrium with untritiated isocitrate.

Results.—Two methods were adopted for the preparation of quinic acid-6-T. In method (*a*) 5-dehydroshikimate was converted to quinate in the presence of tritiated water with NADH and the *A. aerogenes* enzyme preparation. The specific activity of the citric acid finally isolated was about half that of the tritiated water. If it is assumed that the oxidation of quinic to citric acid takes place without loss of tritium and that quinic acid can be labeled at only the one position, then a relatively small (twofold) discrimination against the tritium of the medium occurred. Discriminations as high as 40-fold have been observed.[24] In order to establish that this was characteristic of the 5-dehydroquinate dehydratase reaction, it would be necessary to show that this enzyme was present in a rate-limiting amount in the reaction mixture. In method (*b*) the attempt was made to bring quinic acid into equilibrium with tritiated water through the enzyme-catalyzed cycle of oxidation, dehydration, hydration, reduction. Despite the long period of equilibration, the specific activity of the derived citric acid was only half that of the tritiated water employed.

Although the oxidation of quinic to citric acid was not quantitative, the specific activities of the two acids were in close agreement. It follows that position 4 of quinic acid was not significantly tritiated. If nonenzymatic tritiation had taken place at the 5-dehydroquinate stage, labeling on both C-4 and C-6 (adjacent to the keto group) would be expected.

Comparison of citric acid-4-T with tritiated citric acids prepared by other methods:

Investigations in a number of laboratories into the action of aconitate hydratase have shown that three types of isotopic labeling of the four methylene hydrogens of citric acid may be distinguished: (1) Of the two hydrogens on the oxaloacetate-derived methylene group of citrate, one remains attached to the carbon atom and appears on C-2 of *cis*-aconitate and *threo*-D_s-isocitrate when citrate is acted upon by aconitate hydratase. When the isocitrate is oxidized by $NADP^+$ in the presence of isocitrate dehydrogenase to give α-ketoglutarate, this hydrogen is transferred to the 4*P* position (the *A* side)[11, 26] of the coenzyme-reduced nicotinamide ring.[6, 27, 28] (2) The other hydrogen of this methylene group passes to water when the substrate is brought to isotopic equilibrium with aconitate hydratase. Conversely, hydrogen from the water passes to this position of citrate, but none is incorporated into *cis*-aconitate. In the interconversion of citrate and isocitrate, not all of the molecules pass through the free *cis*-aconitate stage. Hydrogen from the hydratase-exchangeable position of citrate may pass to the C-3 position of isocitrate, and vice versa.[21, 29] The total replacement of hydrogen observed on isotopic equilibration is thus the result of repeated cycling of the substrates. (3) The hydrogens on the acetate-derived methylene group appear on C-4 of α-ketoglutarate when citrate is acted upon by the coupled system aconitate hydratase and isocitrate dehydrogenase.[5]

The results of comparing samples of citric acid-4-T (tests), prepared from quinic acid-6-T, with tritiated citric acids labeled in the above three ways (controls) are shown in Table 1. Both test samples behaved almost precisely as the citric acid prepared from unlabeled *threo*-D_s-isocitrate and tritiated water: nearly all of the tritium was released to the water on prolonged incubation with aconitate hydratase. The citric acids prepared from isocitrate-2-T or -3,4,4-T behaved otherwise: less than 10 per cent of the radioactivity was so released. When the residues from the equilibrium reaction were enzymatically converted to α-ketoglutarate and the α-ketoglutarate converted to glutamate, 79 and 86 per cent of the remaining radioactivity terminated in NADPH and glutamate, respectively. It follows that the maximum fraction of the test citric acids labeled in the acetate-derived methylene is 0.02 per cent and in the oxaloacetate-derived methylene, at the position not associated with the aconitate hydratase exchange reaction, is 2 per cent.

A further test was performed on a sample of citric acid-4-T (from 5-dehydroshikimic acid) with use of the coupled aconitate hydratase–isocitrate dehydrogenase system. The dehydrogenase was present in such a quantity that the rate of increase in absorbancy at 340 mμ was determined by the aconitate hydratase reaction. Throughout the reaction the ratio of tritium released to the water to NADPH formed was essentially constant. This result was typical of experiments performed on citric acid obtained by exchange labeling with tritiated water and aconitate hydratase. The same result has also been observed with various preparations of the hydratase. The fraction of tritium appearing on the C-3 position of α-ketoglutarate when test and control samples were acted upon by the coupled system varied from 40 to 17 per cent for reasons which are not at present understood.

Discussion.—The above results may be summarized as follows: The tritium of citric acid-4-T, obtained from enzymatically labeled quinic acid-6-T, is released to the water when the citric acid is acted upon by aconitate hydratase. Since the portion of the molecule derived from oxaloacetate is known to bear the hydrogen

TABLE 1

EQUILIBRATION OF TRITIATED CITRIC ACIDS WITH ACONITATE HYDRATASE

Tritiated citric acids	Total radioactivity (water + residue), cpm	Distribution of Counts: % Into water	% Into residue: Total	% Into residue: Into NADPH	% Into residue: Into glutamic acid
Tests: citric acid-4-T derived from					
(*a*) 5-Dehydroshikimate	23,323	97	3	0	0.01
(*b*) Quinate	36,640	95	5	1.5	0.015
Controls: tritiated citric acids prepared from					
(1) Isocitrate + T_2O	24,439	95	5	0	0.65
(2) Isocitrate-2-T	37,043	5	95	75	0.30
(3) Isocitrate-3,4,4-T	8,849	8	92	0	79.0

Samples of quinic acid-6-T (V) were prepared from the compounds indicated (methods *a* and *b*) with the aid of 5-dehydroquinate dehydratase and quinate dehydrogenase, and these samples were oxidized to citric acid-4-T (VII). The three types of tritiated citric acids employed as controls were prepared from labeled or unlabeled isocitrate by prolonged incubation with aconitate hydratase. Samples 1 and 2 bear tritium at the oxaloacetate-derived end of the citric acid molecule. Most of the tritium on C-3 of isocitrate-3,4,4-T should have passed to the water, giving citric acid labeled at the end of the molecule not involved in the aconitate hydratase reaction.

Each type of citric acid (0.08–0.25 μmoles) together with buffer (triethanolamine-HCl, 0.1 *M*, pH 8.1) and aconitate hydratase (0.01 units) was maintained at 30° for 9 hr (final volume 1.0 ml). The water of each reaction mixture was separated by sublimation *in vacuo* and the dried residue freed of easily exchanged tritium by adding water and performing a further sublimation. The residue from each incubation was treated with additional aconitate hydratase (0.01 units) and isocitrate dehydrogenase (0.1 mg) in the presence of NADP (0.3 μmoles) and in a final volume of 1.0 ml. The reduction of NADP was followed by measuring the change in absorbancy at 340 mμ. When the reaction was complete, the radioactivity of a sample was counted, and another sample was filtered through a column of octanol-treated charcoal (1.5 × 0.6 cm) which was subsequently washed with excess water. Since the combined effluent and washes showed no absorbancy at 340 mμ, all the NADPH had been adsorbed by the charcoal. The difference in radioactivity between this material and the residue is taken as the NADPH-associated radioactivity. The remainder of the charcoal-treated solution was incubated with glutamate dehydrogenase (1.5 units), NADH (0.2 μmoles), and ammonium acetate (150 μmoles) in order to convert the α-ketoglutarate into L-glutamate (final volume 3 ml). Additional glutamic acid (10 μmoles) was added and the entire reaction mixture was chromatographed on a Dowex 1 acetate column (elution with 0.5 *N* acetic acid). Quantitative recoveries of glutamic acid were obtained. The radioactivity in this material represents the tritium derived from the methylene group of citrate not involved in the aconitate hydratase reaction. The significant fraction of the radioactivity which cannot be assigned to any of the C-H positions of citric acid in the control samples 2 and 3 is considered to represent contamination with the isomers of isocitric acid that are not substrates for aconitate hydratase. The preparations were shown to be free from the *threo*-DS-isomer by assay with isocitrate dehydrogenase. Thus, the radioactivity found in NADPH and glutamic acid was derived from the citric acid present.

exchangeable by aconitate hydratase,[6] the stereochemical course of citric acid biosynthesis is established in absolute terms. The assignment may be expressed in several alternative ways: (*a*) Carbon atoms 6, 3, 4, and 5 of citric acid are derived from oxaloacetate and 1 and 2 from acetyl-CoA (see formulas I and II). (*b*) If the axis of the center carbon atom and its attached —COOH group is equated with the axis of the body so that the —COOH group is at the feet, and if the —OH is behind the body, then the acetyl-CoA-derived —CH_2COOH group is to the right front, and the oxaloacetate-derived —CH_2COOH group to the left front. (*c*) In the formation of citrate from oxaloacetate, the side of the carbonyl group attacked by acetyl-CoA is opposite to the side attacked by NADH in the formation of L-malate.[30]

This conclusion is based on a direct correlation with D(+)-glyceraldehyde: The configuration of quinic acid relative to D-glyceraldehyde is firmly established (the evidence has been reviewed elsewhere[31]). Although the study involves enzymatic labeling of quinic acid, the position of labeling is known from the known chemistry of the enzymatic substrates. It follows that, in setting forth the sequence of reactions involved in the correlation, no assumptions are required concerning the steric course of any reaction. The absolute configuration follows, since the configuration of D-glyceraldehyde is believed to be identical with that adopted in the Emil Fischer convention.[32]

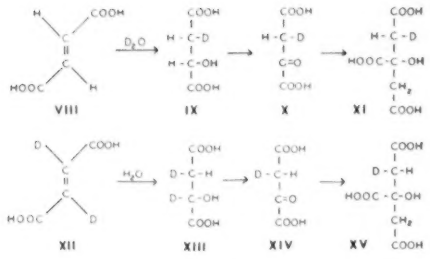

FIG. 3.

Two indirect correlations, based on optical rotation data, have been reported. Unfortunately, the arguments employed lead to opposite conclusions. Thus, in the one study, the enantiomeric forms of oxalocitramalic acid lactone-3,3-D were assigned configurations on the basis of Hudson's lactone rule. Oxidation of the free acids gave the corresponding deuterated citric acids.[5] In the other, a configuration was assigned to (−)-2-chloromethyl-2-hydroxysuccinic acid by means of Brewster's rules.[33] It had previously been shown[3] that asymmetrically labeled citric acid could be obtained from this compound with the aid of sodium cyanide-C^{14}. Both groups of investigators arrived at the assignment reached in the present paper. However, the latter group base their conclusion on a misreading of the experiments of Wilcox *et al.*[3] Given the premise that Brewster's rules apply, their conclusion must be reversed. We are indebted to Dr. Hirschmann for drawing our attention to this error.

The configuration at the oxaloacetate-derived methylene group of citric acid has been correlated with the fumarate hydratase (E.C.4.2.1.2, fumarase) reaction by Englard.[6] The *trans*-nature of this reaction has been established by Gawron *et al.*[33] and by Anet.[34] The conclusion that the C-4 methylene group of citric acid is derived from oxaloacetate leads to the summary of Englard's experiments (Fig. 3). In terms of the anterior (*A*), posterior (*P*) convention of Hirschmann,[11] citric acid-4(*A*)-D (XI) arises from fumaric acid-2,3-D (VIII) by way of L-malic acid-2,3(*A*)-D and oxaloacetic acid-3(*A*)-D, etc. When the two citric acids were treated with aconitate hydratase and isocitrate dehydrogenase, the deuterium from fumaric acid-2,3-D (VIII) appeared in NADPH, while the deuterium from D_2O did not. The hydrogen which exchanges with the hydrogen of the medium in the presence of aconitate hydratase is therefore attached to C-4 in the posterior position (see II and XVI). The stereochemistry of the aconitate hydratase reaction follows: The addition of T_2O to *cis*-aconitate (XVII) to give citrate-4(*P*)-T (XVI) or to give *threo*-D_s-isocitrate-3-T (XVIII) is *trans* in both cases. A given double-bond carbon atom is always attacked from the same side. Gawron *et al.*[33] have already noted that these conclusions follow if C-1 and C-2 of citrate are acetate-derived.

The stereochemistry of the 5-dehydroquinate dehydratase reaction may also be inferred. Thus, if the enzymatic hydration of 5-dehydroshikimate (III) is *cis*, then citric acid-4(*P*)-T (XX) is obtained via the intermediates 5-dehydroquinic

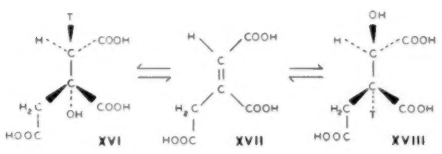

FIG. 4.

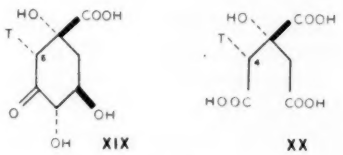

FIG. 5.

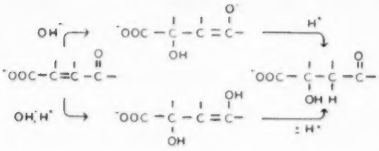

FIG. 6.

acid-6(*P*)-T (XIX) and quinic acid-6(*A*)-T. This appears to be the first example of enzymatic *cis* addition to a double bond.

In considering the mechanism of the 5-dehydroquinate dehydratase reaction, it may first be noted that the position of OH^- attack on 5-dehydroshikimate (III) is β to the C-5 keto group. Such an addition is in accordance with experience for nonenzymatic reactions: the keto group shows a stronger directing influence than the carboxyl ion. The addition process need not be formulated as a concerted addition to the double bond. By analogy with the behavior of unconjugated double bonds, an addition of this type might be expected to be *trans*. If the addition to the α and β positions takes place in two separate stages, then the stereochemistry will depend entirely upon the arrangement of acceptor-donor groups on the enzyme. Examples of such two stage mechanisms are shown in Figure 6. The nucleophylic addition of methoxy to *trans*-dibenzoylethylene has been formulated in terms analogous to the first mechanism. The methoxyl addition is here the rate-determining step.[35]

Summary of Conclusions.—(1) If a molecule of biosynthetically formed citric acid is viewed from the side opposite the middle COOH group, then moving from —OH in a counterclockwise direction the first $—CH_2COOH$ is derived from acetyl-CoA, and the second from oxaloacetate. (2) The enzymatic addition of water to *cis*-aconitate to give citrate is *trans*. (3) The enzymatic addition of water to 5-dehydroshikimate to give 5-dehydroquinate is *cis*.

Nomenclature: The Hirschmann rules[11] for designating the various atoms about a *meso* carbon atom have been used in this paper wherever appropriate. The various compounds in which tritium or deuterium has replaced protium, however, are asymmetric in the conventional sense and the configurations at the asymmetric centers may be fully specified by the *R/S* system.[36] Since the *A/P* system is linked to the *R/S* system, and since protium has the lowest priority of any substituent, *replacement of protium in the* P *position by deuterium or tritium necessarily gives* R *asymmetry, and replacement in the* A *position gives* S *asymmetry.* It follows that citric acid-4(*P*)-T is (2*R*,3*R*)-2-tritiocitric acid (sub-rules 1 and 4) and quinic acid-6(*A*)-T is (6*S*)-6-tritioquinic acid or (1*R*,2*S*,3*R*,4*S*,5*R*)-1,3,4,5-trihydroxy-2-tritio*cyclo*hexane-1-carboxylic acid (sub-rules 1 and 4). Quinic acid itself is (1*R*,3*R*,4*S*,5*R*)-1,3,4,5-trihydroxy*cyclo*hexane-1-carboxylic acid (C-3 and C-5 by sub-rule 1, C-1 and C-4 by sub-rule 2). (We are indebted to Dr. Cahn for his comments on the application of the *R/S* system to the quinic acids.) *Threo*-D_s-isocitric acid is (2*R*,3*S*)-isocitric acid and tritium substitution does not alter the *R/S* labels. The fluorocitric acid derived biosynthetically from fluoroacetate is either (2*S*,3*S*)- or (2*R*,3*S*)-2-fluorocitric acid.[31]

The asymmetric centers of citric acid-4(*P*)-T may also be defined by extending carbohydrate-amino acid nomenclature in the manner used for the isocitric acids.[37] The procedure leads to the names D_sD_6-2-tritiocitric acid or *erythro*-D_s-2-tritiocitric acid (the COOH attached to C-3 is here disregarded).

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[30] P. F. V. Ward and R. A. Peters, [*Biochem. J.*, **78**, 661 (1961)] have shown that a single fluorocitric acid accumulates *in vitro* in rabbit kidney poisoned with monofluoroacetate. Two diastereoisomers are theoretically derivable through the action of citrate synthase. Such an accumulation would arise if the enzyme is able to catalyze the replacement of only one of the two nonequivalent α-hydrogens of fluoroacetyl-CoA in the condensation with oxaloacetate.
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*A STOCHASTIC MODEL FOR THE INTERPRETATION OF CLINICAL TRIALS**

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1. *Introduction.*—There are several diseases in which possibly recurrent phases may be distinguished. Different investigators have proposed Markovian models to describe data dealing with the time-dependent phenomena associated with these diseases. We mention in particular the work of Fix and Neyman on cancer,[1] the

work of Marshall and Goldhamer on the epidemiology of mental disease,[2] and the work of Alling on tuberculosis.[3] All of these assume that the distribution of time spent in an occurrence of a particular phase is negative exponential. In the following paper we present a semi-Markov model for data analysis, in which it is possible to consider any distribution for stay in a given phase. The theory has been applied to data on victims of both acute myelocitic and acute lymphocytic leukemia who have received experimental drug therapy. In our application we have found that gamma distributions give a convenient representation of the relevant probability densities. Hence, any Markov theory will not be sufficient for the study of the statistics relating to leukemia. We feel that the semi-Markov model presented here has wide applicability to many clinical situations.

The advantage of having a model is that it guides the investigator in the types and kinds of data to collect. Further, a model serves as a convenient frame of reference for posing questions and suggesting further experiments. Too often the "effectiveness" of a treatment is measured by the "success ratio." It is clear that other factors are also important; e.g. (using the terminology of the acute leukemia study), time in a remissive state, time to reach a remissive state, degree of toxicity, time to failure (if ailment is a fatal one), time to complete cure, etc.

2. *Formulation of the Model.*—The health of a patient can be characterized at any instant of time by being in one of a finite number of states. In the clinical terminology, the patient may be in a relapse state, a remissive state, toxic state, etc. These remissive and relapse states may be further classified by the degree of remission or relapse and also by how many and what kinds of relapse or remissions preceded the present state. In addition to these transient states, the patient may have entered a terminal (or absorbing, in the language of Markov processes) state such as failure, failure from other causes, cure, or patient lost.

We shall let T denote the set of transient states and A the set of absorbing states. Also we define:

$U_i(t)$ = probability of being in state i at time t;

$q_i(t)$ = probability density function for a single time in state $i \epsilon T$;

$Q_i(t)$ = $\int_t^\infty q_i(x)dx$;

$\omega_i(t)dt$ = probability of leaving state i ($i \epsilon T$) during the time interval $(t, t + dt)$;

p_{ij} = probability of passing from state i to j, conditional upon leaving i (by convention we set $p_{ii} = 0$ for $i \epsilon T$).

It will be convenient to define the vector and matrix analogues of the above quantities. For this purpose we let

$\mathbf{U}_T(t)$ = column vector of $U_i(t)$, $i \epsilon T$;

$\mathbf{U}_A(t)$ = column vector of $U_i(t)$, $i \epsilon A$;

$\mathbf{q}(t)$ = $(\delta_{ij} q_i(t))$, where δ_{ij} is the Kronecker delta;

$\mathbf{Q}(t)$ = $(\delta_{ij} Q_i(t))$;

$\boldsymbol{\omega}(t)$ = column vector of $\omega_i(t)$; $\mathbf{U}^0 = (U_i(0)\delta_{ij})$, $i \epsilon T$;

$$\mathbf{P} = \begin{matrix} \\ A \\ T \end{matrix} \begin{matrix} \begin{matrix} A & T \end{matrix} \\ \begin{bmatrix} \mathbf{I} & \mathbf{O} \\ \mathbf{R} & \bar{\mathbf{P}} \end{bmatrix} \end{matrix}.$$

As a further convention, we denote the Laplace transform of any time-dependent function by the same function with argument s and an asterisk; i.e., $\omega_i^*(s) = \int_0^\infty e^{-st}\,\omega_i(t)dt$, $\boldsymbol{\omega}^*(s) = \int_0^\infty e^{-st}\,\boldsymbol{\omega}(t)dt$.

Using familiar arguments in the theory of semi-Markov processes,[4] one can obtain the following equations for $\boldsymbol{\omega}^*(s)$ and $\mathbf{U}_T^*(s)$:

$$\boldsymbol{\omega}^*(s) = \mathbf{q}^*(s)\mathbf{U}^0 + \mathbf{q}^*(s)\bar{\mathbf{P}}'\boldsymbol{\omega}^*(s) \tag{1}$$

$$\mathbf{U}_T^*(s) = \mathbf{Q}^*(s)\mathbf{U}^0 + \mathbf{Q}^*(s)\bar{\mathbf{P}}'\boldsymbol{\omega}^*(s) \tag{2}$$

which yield the solutions

$$\boldsymbol{\omega}^*(s) = [\mathbf{I} - \mathbf{q}^*(s)\bar{\mathbf{P}}']^{-1}\mathbf{q}^*(s)\mathbf{U}^0 \tag{3}$$

$$\mathbf{U}_T^*(s) = \mathbf{Q}^*(s)[\mathbf{I} - \bar{\mathbf{P}}'\mathbf{q}^*(s)]^{-1}\mathbf{U}^0. \tag{4}$$

Let $U_A(t)$ denote the probability of being in an absorbing state at time t. Then we have

$$U_A^*(s) = \frac{1}{s} - \sum_{i\epsilon T} U_i^*(s) \tag{5}$$

from which the moments of the time to reach absorption can easily be calculated by noting that the Laplace transform of the survivorship function, i.e., $G(t) = \Pr.\{\text{failure time} > t\}$ is

$$G^*(s) = \sum_{i\epsilon T} U_i^*(s) = \mathbf{1}'\mathbf{U}_T^*(s) \tag{6}$$

where $\mathbf{1}$ is a column vector of appropriate dimension having all elements unity.

For the purpose of writing the moments associated with $G(t)$, define

$$m_i(k) = \int_0^\infty t^k q_i(t)dt,\ \mathbf{M}(k) = (m_i(k)\delta_{ij})$$

$$\tau = \lim_{s\to 0_+} \omega^*(s) = (\mathbf{I} - \bar{\mathbf{P}}')^{-1}U^0.$$

Note that τ_i is the expected number of times state i is visited. Then the mean and variance of the time to reach absorption are

$$E(t) = \lim_{s\to 0_+} G^*(s) = \mathbf{1}'\mathbf{M}(1)[\mathbf{I} - \bar{\mathbf{P}}']^{-1}U^0 = \mathbf{1}'\mathbf{M}(1)\tau \tag{7}$$

$$\sigma^2 = -\lim_{s\to 0+}\left\{2\frac{dG^*(s)}{ds} + [G^*(s)]^2\right\} = \mathbf{1}'\boldsymbol{\Sigma}\tau + \mathbf{1}'\mathbf{M}(1)\{[2(\mathbf{I} - \bar{\mathbf{P}}')^{-1} - \mathbf{I} - \tau\mathbf{1}']\mathbf{D}(\tau)\}\mathbf{M}(1)\mathbf{1} \tag{8}$$

where

$$\boldsymbol{\Sigma} = \mathbf{M}(2) - \mathbf{M}^2(1)$$

$$\mathbf{D}(\tau) = \begin{pmatrix} \tau_1 & & 0 \\ & \tau_2 & \\ & & \ddots \\ 0 & & \tau_n \end{pmatrix} \tag{9}$$

Relatively simple recursive relations can be derived for the higher moments.

In addition to the results given so far, it is possible to develop a theory in which the p.d.f. $q_i(t)$ is replaced by $q_{ij}(t)$, i.e., in which the time spent in any state depends either on the succeeding or the following state. However, the results do not have the simplicity of those of equations (7) and (8). They will appear in a forthcoming publication. Another statistic of some interest in drug evaluation is the total time spent in a given transient state. This is important in the leukemia study, since it is desirable to prolong a patient's life in a condition of remission rather than in a condition of active illness. Let us consider a single remissive state i and partition the transition matrix $\mathbf{P}$ as

$$\mathbf{P} = \begin{array}{c} \begin{matrix} A & i & T-i \end{matrix} \\ \begin{bmatrix} \mathbf{I} & \mathbf{O} & \mathbf{O} \\ \boldsymbol{\delta} & \mathbf{O} & \boldsymbol{\beta} \\ \boldsymbol{\gamma} & \boldsymbol{\alpha} & \boldsymbol{\Pi} \end{bmatrix} \begin{matrix} A \\ i \\ T-i \end{matrix} \end{array}. \tag{10}$$

Then the probability of entering state i at least once starting from state j is

$$\begin{aligned} h_{ji} &= [(\mathbf{I} - \boldsymbol{\Pi})^{-1}\boldsymbol{\alpha}]_j \quad j \neq i \\ h_{ii} &= \boldsymbol{\beta}(\mathbf{I} - \boldsymbol{\Pi})^{-1}\boldsymbol{\alpha}. \end{aligned} \tag{11}$$

The first two moments of the total sojourn time in state i conditional on starting from state j are

$$\left.\begin{aligned} \mu_{ji}(1) &= \frac{m_i(1)}{1 - h_{ii}}\,\delta_{ij} + \frac{m_i(1)h_{ji}}{1 - h_{ii}}\,(1 - \delta_{ij}) \\ \mu_{ji}(2) &= \left[\frac{m_i(2)}{1 - h_{ii}} + \frac{2m_i^2(1)h_{ii}}{(1 - h_{ii})^2}\right][\delta_{ij} + h_{ji}(1 - \delta_{ij})]. \end{aligned}\right\} \tag{12}$$

3. *Applications to Acute Leukemia Clinical Data.*—A large number of clinical trials were conducted by the Acute Leukemia Group B (Frei[5]) on patients having acute leukemia. In this section we will illustrate the application of our model to a portion of these data. A more complete discussion of the data will be given in another publication.

All patients entered these clinical trials while in a state of relapse. The data examined in this paper were for patients who were initially given Methotrexate (MTX). The patients either have responded and expired, or eventually reached a remissive state. A distinction was made between a partial and complete remission. If the patients did not show remission within the first 6 weeks, the MTX therapy was stopped, a period of two weeks was allowed to elapse, at the conclusion of which the surviving patients who were still in a state of relapse were given 6-mercaptopurine (6-MP). Further, if a patient who was in remission entered a relapsed state, the therapy was changed to the alternate drug (MTX or 6-MP). In this paper we will apply the model to data from 26 patients who achieved a state of remission within 8 weeks of entering these clinical trials. In our application we will be mainly interested in the distribution of the time to failure. Since, by definition, a remissive state is always followed by a relapse state, we will combine the sojourn time in a relapse state with the sojourn time of the remissive state which immediately preceded it. Another characteristic of this application is that the data

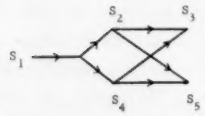

FIG. 1.—State diagram for a model of leukemia.

indicate different sojourn time distributions which depend on the number of times the patient has been in the state. With these characteristics of the process in mind, we define the six states: S_0: failure (death); S_1: initial relapse state (condition of patient on entering study); S_2: first partial remission (also includes subsequent relapse); S_3: second partial remission (includes subsequent relapse); S_4: first complete remission (includes subsequent relapse); S_5: second complete remission (includes subsequent relapse).

The communication among states may be summarized in the diagram above where all states connect with S_0.

An investigation of the distribution of sojourn times within the various transient states showed that with the exception of the initial state, these distributions can be well approximated by gamma distributions, i.e.,

$$\varphi(t) = \lambda e^{-\lambda t}(\lambda t)^{a-1}/\Gamma(a),\ t \geq 0,\ a > 0.$$

The parameters (a, λ) were estimated from the data by the method of maximum likelihood with the aid of the convenient tables of Wilk, Gnanadesikan, and Huyett.[6] Table 1 summarizes the results of these calculations.

TABLE 1

SAMPLE MEANS, VARIANCES, AND MAXIMUM LIKELIHOOD ESTIMATES OF THE PARAMETERS OF GAMMA DISTRIBUTION*

State	a	$\hat{\lambda}$	m(1)	$\hat{\sigma}^2$	Sample size
S_2 (first P. R.)	2.3	0.101	23.1	189	14
S_3 (second P. R.)	2.9	0.122	23.6	215	7
S_4 (first C. R.)	4.7	0.132	35.6	310	27
S_5 (second C. R.)	15.5	0.463	33.6	90	7

* These estimates are based on all patients who went into a remission state whether they left the initial relapse state within eight weeks or after.

It remains to obtain estimates of the transition probabilities **P** before the formulas of the preceding section can be applied. The observed relative frequencies (conditional on going to a remission state) observed in these clinical trials were used as estimates of the (p_{ij}). The numerical results are:

$$\mathbf{P} = \begin{array}{c} \\ S_0 \\ S_1 \\ S_2 \\ S_3 \\ S_4 \\ S_5 \end{array} \begin{array}{c} \begin{array}{cccccc} S_0 & S_1 & S_2 & S_3 & S_4 & S_5 \end{array} \\ \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 10/26 & 0 & 16/26 & 0 \\ 5/10 & 0 & 0 & 3/10 & 0 & 2/10 \\ 1 & 0 & 0 & 0 & 0 & 0 \\ 9/16 & 0 & 0 & 4/16 & 0 & 3/16 \\ 1 & 0 & 0 & 0 & 0 & 0 \end{bmatrix} \end{array}$$

The estimate of the transition matrix **P** and the parameters of the gamma distribution summarize the relevant information with the exception of the sojourn time distribution for the initial relapse state (S_1). Here the distribution is complicated by being truncated at the end of eight weeks. Since the data are summarized in units of a week, this distribution (conditional on a patient reaching a remissive state) was assumed to be a discrete distribution where p_n denotes the

probability of entering a remissive state after n weeks in the initial relapse state. The observed relative frequencies were taken as the estimates for p_n and were:

$n =$	2	3	4	5	6	7
$p_n =$	[2/26	6/26	8/26	5/26	4/26	1/26]

A check on the model can be obtained by comparing the sample mean and variance of the time to reach failure with the theoretical formulas given in (7) and (8). The numerical results are:

	Data	Mode
Mean	48.4	49.7
Variance	515.	525.

We now turn our attention to estimating the distribution of the time to reach the failure state. The probability density function for those patients reaching a remissive state can be calculated using (6). After some reduction, the result is:

$$g_0(t) = p_{20}p_{12}[\varphi_1 * \varphi_2] + p_{30}[p_{12}p_{23}(\varphi_1 * \varphi_2 * \varphi_3) + p_{14}p_{43}(\varphi_1 * \varphi_3 * \varphi_4)] + p_{40}p_{14}[\varphi_1 * \varphi_4] + p_{50}[p_{12}p_{25}(\varphi_1 * \varphi_2 * \varphi_5) + p_{14}p_{45}(\varphi_1 * \varphi_4 * \varphi_5)].$$

(The notation $\varphi_1 * \varphi_2$ denotes the convolution of $\varphi_1(t)$ and $\varphi_2(t)$, where $\varphi_1(t)$ denotes the frequency function of the initial relapse state.)

Note that $g_0(t)$ is made up of a mixture of distributions which involve convolutions of gamma distributions. For our purposes, the approximation of convolutions of gamma distributions by a gamma distribution with the same first two moments was deemed a sufficient approximation. Figure 2 contains a plot of $G_0(t) = \int_0^t g_0(x)dx$, along with the sample data.

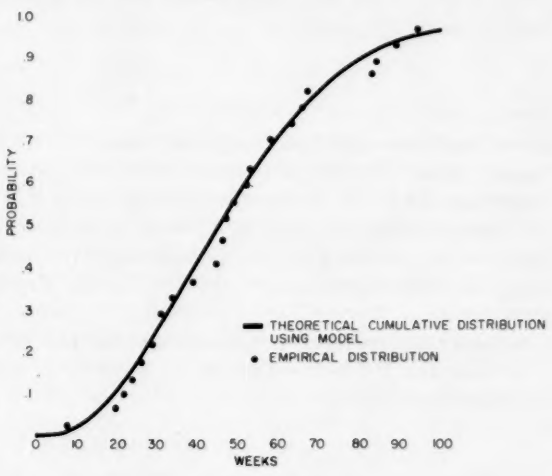

FIG. 2.

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ON DIFFERENTIAL SYSTEMS, II

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In the study of the "general equivalence problem," one of the principal tools is the use of the structure function to reduce the group. (For a discussion of this technique for the case of G-structures, cf. ref. 3; the general setting, where the structure groups are allowed to vary from point to point, is the notion of differentiable groupoid of Ehresmann.[1] We hope to discuss the foundations of the general theory in the near future.) Here we shall present some techniques applicable in the case that our structure is subordinate to a nonintegrable differential system. More precisely, we shall assume that the Lie algebra of the structural group at each point has the form

$$\begin{pmatrix} \tilde{g} & 0 \\ h & g \end{pmatrix}$$

where the lower-right-hand block corresponds to the invariant subspace V_1 of the tangent space V (and g is the restriction of the structure algebra to V_1) where $\tilde{g}$ is the induced action of the structure algebra on V/V_1 and where we assume that the structure function $\rho: V_1 \wedge V_1 \to V/V_1$ of the differential system is surjective. (This is our nonintegrability assumption.) In particular, $\tilde{g}$ is a homomorphic image of g and we have $\tilde{Y} \circ \rho = \rho \circ \wedge^2(Y)$ for any $Y \in g$. We wish to describe how to cut down h.

Setting the structure function constant has the following effect on h:

1. For any $X \in h$, $X \circ \rho$ lies in $V_1 \otimes \wedge^2(V_1^*)$. Setting the structure function constant implies that

$$X \circ \rho \text{ lies in } \partial(g \otimes V_1^*) \tag{1}$$

(in the Spencer complex). Notice that if $g^{(1)} = 0$ (i.e., $H^{2,0} = 0$) then we have a *unique* S with

$$X \circ \rho = \partial S. \tag{2}$$

(This is useful when g is a "small" subalgebra of Hom (V_1, V_1), in particular if V_1 is of small codimension >2.)

2. To each $v \in V_1$ and $X \in h$ associate $(v \lrcorner \rho) \circ X \in$ Hom $(V/V_1, V/V_1)$. Then setting the structure function constant implies that

$$(v \lrcorner \rho) \circ X \in \tilde{g} \tag{3}$$

so that X gives $T \in \tilde{g} \otimes V_1^*$. (This is useful when V_1 is of large codimension.)

3. If $g^{(1)} = 0$ (so that (2) holds) then we have

$$\widetilde{S(v)} = T(v)$$

for all $v \in V_1$.

As an example of the first technique, see reference 2 where we prove that a generic n-distribution in $n + n(n-1)/2$ space is of finite type.

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DEVELOPMENTAL CHANGES AND HETEROGENEITY OF LACTIC AND MALIC DEHYDROGENASES OF HUMAN SKELETAL MUSCLES AND OTHER ORGANS

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Lactic dehydrogenase (LDH), the enzyme catalyzing the reversible reduction of pyruvate, is ubiquitous in mammalian tissues and has been shown to exist in 5 distinct molecular forms within the tissues of each mammal that has so far been studied. These isoenzymes of LDH may be separated by electrophoresis on different suitable media,[1-8] column chromatography,[9] or by coenzyme analogue specificity using nicotinamide adenine dinucleotide (NAD).[10] They differ in their catalytic and immunologic properties, amino acid compositions, and also in certain physical characteristics.[11, 12] It has been demonstrated that each LDH isoenzyme molecule is formed from four polypeptide subunits of equal size and that the subunits are divisible into two distinctive varieties by electrophoresis.[11, 12] The LDH isoenzymes are designated respectively as LDH_1 for the most rapidly migrating anodal form to LDH_5, the cathodal slowly moving variety at pH 8.6. Both LDH_1 and LDH_5 are composed of four identical subunits, each of a different type. LDH_2, LDH_3, and LDH_4 are made up of the two types of subunits in various combinations. They are thus hybrid forms.

The two pure types of LDH have also been designated as M (muscle or LDH_5) and H (heart or LDH_1) forms.[12] Recently, Kaplan and Cahn[13] reported that the composition of LDH in the various skeletal muscles of the normal chicken differs to a great extent, ranging from almost pure H type to pure M type. The red and white muscle fibers of the guinea pig, rabbit, and mouse have also been found to differ in respect to LDH isoenzyme composition.[14] In examining the isoenzyme patterns of human skeletal muscles, previous investigators[7, 15] have not taken into consideration the heterogeneous nature of this tissue. In the present paper, evidence is given for the heterogeneity of LDH and malic dehydrogenase (MDH) isoenzyme compositions of human skeletal muscles and various visceral organs.

The changes in isoenzyme pattern in these tissues during development will also be presented.

Materials and Methods.—Preparation of tissue extracts: Various human skeletal muscles and organs were obtained at autopsy within 4–10 hr after death from persons who died due to diseases other than those of neuromuscular origin. (We have evidence, derived from comparing enzyme content and isoenzyme distribution, that muscle obtained at autopsy over this interval of time does not lose much LDH enzyme when compared with freshly biopsied and processed muscle.) The tissue (100–300 mg) was homogenized with cold double-distilled water (1:4 v/v) in a small all-glass Potter-Elvehjem homogenizer for 3–5 min at 5°. Centrifugation of these homogenates was done at 0° and 2,000 *g* for 15 min and the clear supernatant fractions were used for enzyme study.

Separation of LDH and MDH isoenzymes by agar gel electrophoresis: Agar gel electrophoresis on ordinary microscope slides (25 × 75 mm) was carried out in a Spinco-Durrum type of electrophoretic cell by modification of the method of Yakulis and his associates[16] as follows: the support stand was removed from the Durrum electrophoretic cell and the cell was filled to the fluid line with barbital buffer, pH 8.6, of ionic strength 0.05. The slides containing the agar gel were placed on the two wick supports and the connection of the gel with the buffer solution was established by means of pieces of Whatman 3 mm filter paper (1″ × 1″). The agar gel was prepared by dissolving 1 gm of Ion Agar no. 2 (Consolidated Laboratories) in 100 ml of warm barbital buffer, pH 8.6, of ionic strength 0.025 and the gel thickness was maintained constant by pouring in 2

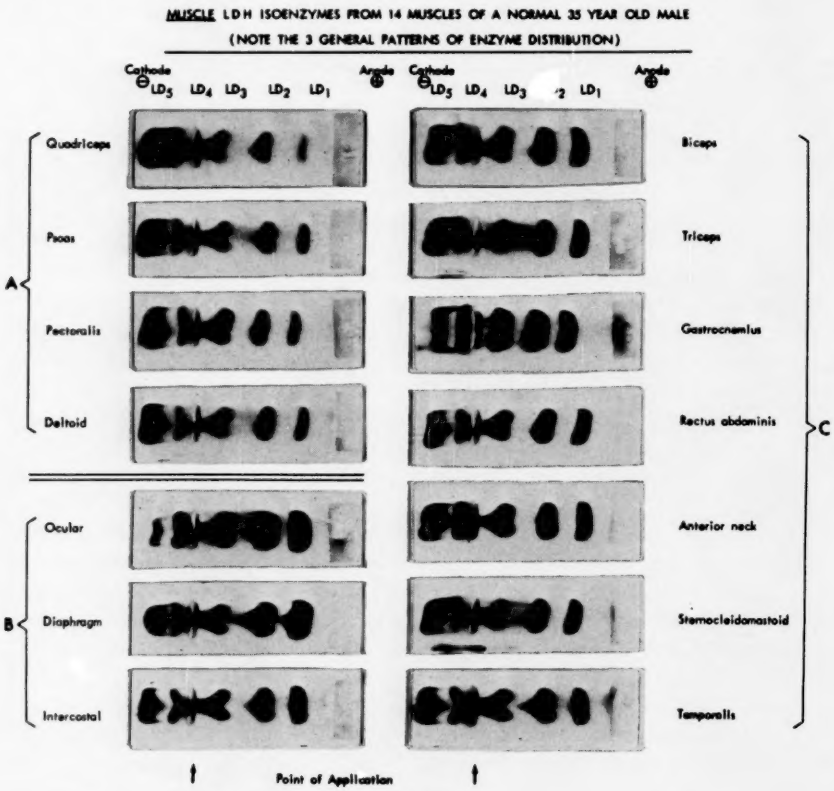

FIG. 1.

ml of hot agar solution to each slide. The agar gel was allowed to set for 5 min and a small groove (1 × 10 mm) was made with a cutter which was fashioned from 2 single-edged razor blades. Ten μl of the tissue extract can be placed in this groove. Six slides could be easily subjected to electrophoresis simultaneously with good separation of the isoenzymes. A potential gradient of 6.5 volts/cm was maintained for 90 min at room temperature.

Localization of LDH and MDH isoenzymes: After electrophoresis, the LDH isoenzymes were localized on the gel by incubating the slides with a substrate medium as described by Yakulis and his associates[16] and modified in the following manner: the slides were placed in a petri dish containing 10 ml of substrate medium which consisted of: phosphate buffer, pH 7.4, 0.1 *M*, 5 ml; sodium *DL*-lactate, 1 *M*, 1 ml; NAD, 10 mg/ml, 0.50 ml; phenazine methosulfate, 1 mg/ml, 0.50 ml; nitro-blue tetrazolium (Nutritional Biochemicals), 1 mg/ml, 2.5 ml; double-distilled water, 0.50. These were then incubated for 90 min at 37° in the dark. The distribution of MDH isoenzymes was determined in a similar manner, except that sodium *DL* malate was used in the substrate medium instead of sodium lactate. After the incubation was over, the slides were washed repeatedly with ethanol:acetic acid:water (5:1:5 v/v), until the background of the gel was clear. These were then covered with filter paper and dried at 37°. After drying, the slides could be stored indefinitely in the dark. They were then photographed and a semiquantitative idea about the proportional distribution of each LDH isoenzyme could be obtained by visual inspection of the stained zones.

Assay of LDH: Total LDH activity of the muscle extracts were determined by the method of Cabaud and Wroblewski.[17]

Results.—LDH isoenzyme patterns of adult human muscle: In Figure 1 are compared the LDH isoenzyme patterns of 14 muscles of a normal adult human male. It can be seen that every muscle examined contains all five LDH isoenzymes but each in somewhat different proportions. A similar set of patterns was also found in another normal adult. In general, these patterns could be grouped into three distinct classes as follows: (*a*) predominant M type (i.e., LDH_5) with a steady decline of activity in the other isoenzymes toward the anodal pole; (*b*) predominant H type (i.e., LDH_1) with a gradual diminution of activity toward the cathodal end of the spectrum; and (*c*) the mixed type of LDH, found in the majority of muscles examined, where nearly equal amounts of enzyme activity are present in all fractions of the isoenzyme spectrum.

From Table 1, it is apparent that the total LDH activity per gm wet weight also varies considerably from one muscle to another.

LDH isoenzyme patterns of muscles obtained from a newborn male child: In Figure 2 are shown the LDH isoenzyme patterns from various muscles of a 2-day old male child. It is evident that in all the muscles examined most of the enzyme activity is of the H type. One can observe the differences that exist between the LDH compositions of muscle from this newborn and from adult muscle by comparing specific muscles in Figures 1 and 2 or in the lower part of Figure 4.

TABLE 1

TOTAL LDH ACTIVITY OF 14 MUSCLES OF A NORMAL HUMAN ADULT

Muscle	Units/gm of wet muscle*	Muscle	Units/gm of wet muscle*
Quadriceps	112,500	Biceps	80,500
Psoas	117,000	Triceps	57,500
Deltoid	64,500	Gastrocnemius	67,000
Pectoralis	98,000	Rectus abdominis	144,000
Ocular	102,000	Anterior neck	58,000
Diaphragm	130,000	Sternocleidomastoid	130,000
Intercostal	40,500	Temporalis	89,500

* Each unit is equivalent to that amount of LDH that would cause a decrease in $O.D._{340}$ of 0.001/min in a reaction mixture of 3 ml volume as described by Wroblewski and La Due.[22]

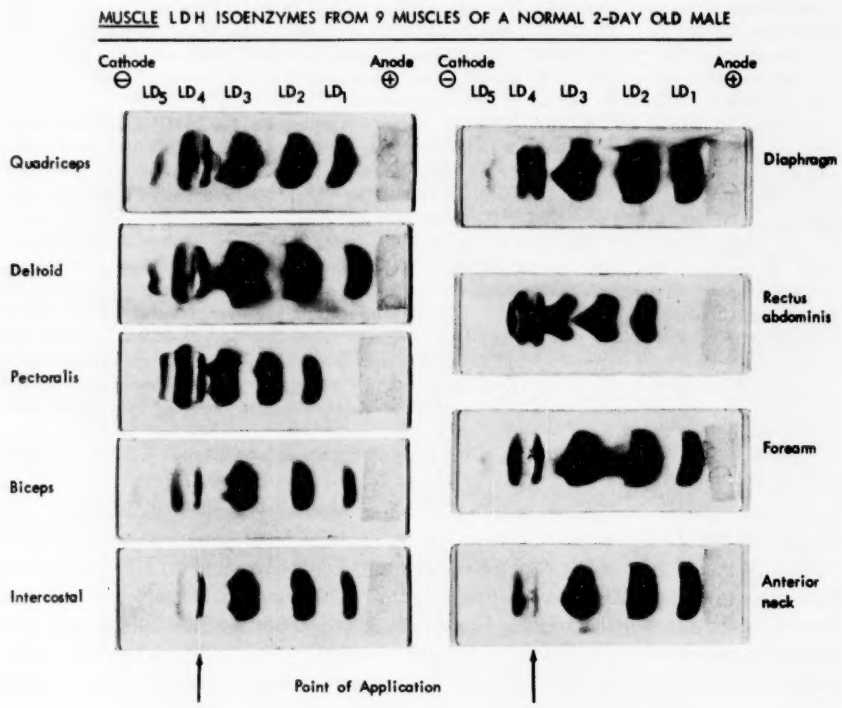

FIG. 2.

MDH isoenzyme patterns of muscles: Contrary to the diverse patterns of the LDH isoenzymes in different muscles, the isoenzymes of MDH show only mild variation in their composition. All the muscles examined contain four MDH isoenzyme bands. The fastest-moving ones (MDH_1 and MDH_2) are very faint in all of them and do not photograph well (Fig. 3). In some of the muscles, as in in group B of Figure 3, the band moving toward the cathode (MDH_4) shows considerable activity. MDH isoenzyme patterns of the muscles from the 2-day-old male child show no characteristic difference when compared to those of the adult, except that MDH_1, MDH_2, and MDH_4 are even fainter in the 2-day-old muscles and only MDH_3 stands out clearly.

Developmental changes of LDH isoenzyme patterns of certain organs: As can be seen in Figure 4, LDH patterns of all of the organs which were examined in a 2-day-old infant show most activity in the fast-moving bands. Only the liver contains more than trace amounts of LDH_5. The greatest changes in isoenzyme patterns during development occur in the liver and some striated muscles. Lesser change takes place in the spleen and none at all in the heart and lung.

Muscle LDH isoenzyme patterns in several myopathies: Biopsy specimens of quadriceps muscle from patients with various myopathies have been analyzed for their LDH isoenzyme compositions. It has been found that in certain forms of myopathy, especially some types of muscular dystrophy, the pattern closely

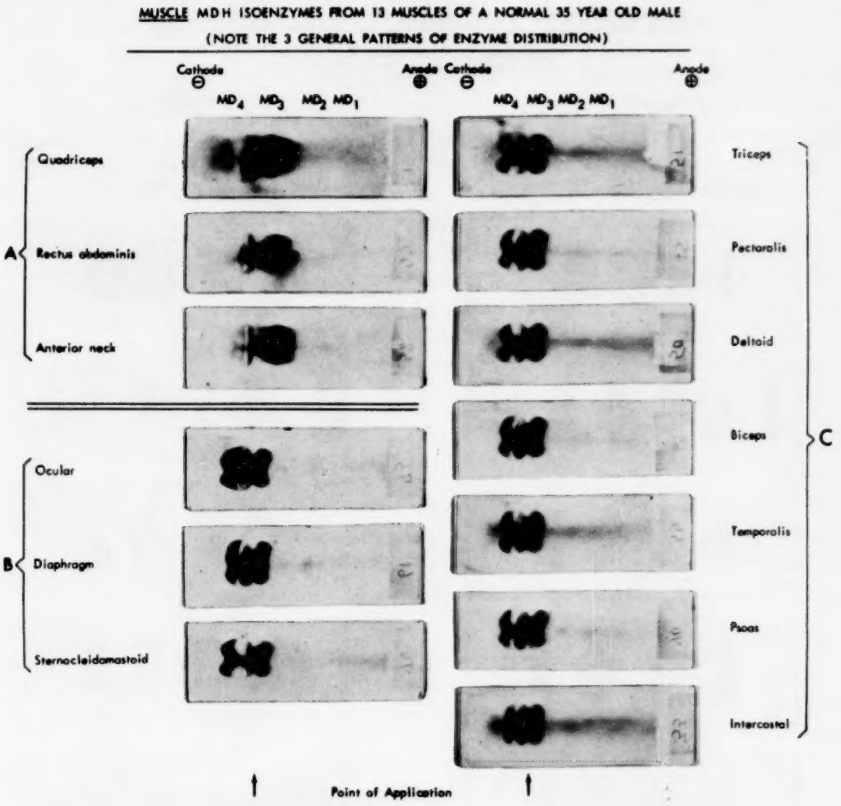

FIG. 3.

resembles that of the fetal quadriceps muscle. Others, however, show no abnormality in LDH composition. Details of this study will be presented in a separate paper.

Discussion.—The data presented in this paper show that in the human subject the enzyme LDH exists in several molecular forms in many body tissues and that the proportions of these forms differ from one tissue to another. Moreover, as previously found in the chicken,[13] the composition of LDH isoenzyme forms varies even in the same tissue, namely, striated skeletal muscle. Important lessons to be learned from these observations are that: (*a*) care must be taken to sample the same muscle each time during a serial investigative study, (*b*) the muscle which has been analyzed should be clearly defined in each report, and (*c*) when analyzing diseased muscle, control samples should be obtained from the *same* muscle of a normal subject.

It is probable that the different isoenzyme patterns which are found in various tissues and organs are reflections of the various metabolic requirements within the specific tissues. If this is the case, then it implies that differing physiological and metabolic roles are being played by various segments of the body musculature.

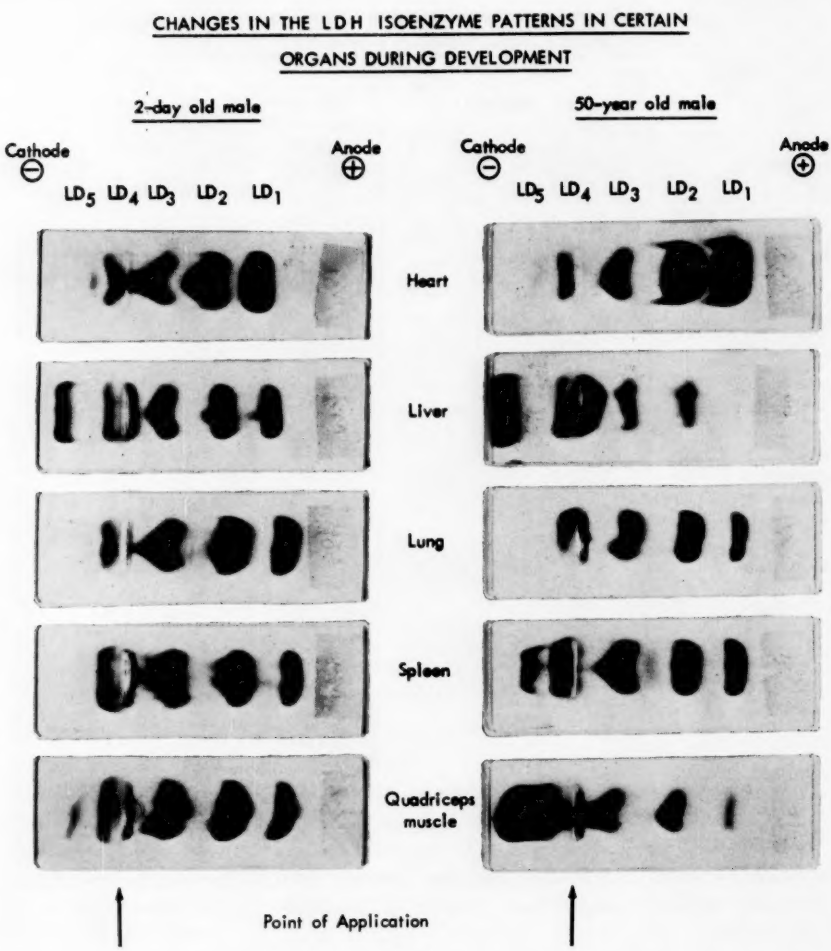

FIG. 4.

Blanchaer and van Wijhe[14] have looked into this matter by examining both red and white portions in several muscles from guinea pigs, rabbits, and mice. They found that red muscle contained all five bands of LDH in nearly equal proportion, whereas in white muscle only LDH_4 and LDH_5 were consistently present. Man does not clearly have red and white muscles as such, and all muscles probably contain a mixture of both red and white fibers. Muscles do, however, vary somewhat in the intensity of their coloration so that the patterns of LDH isoenzymes may reflect the proportional complement of each of these two types of fibers. In addition to the variable intensity of coloration of muscle, which is due to its myoglobin and cytochrome content, red and white fibers also have been shown to have other histochemical[18] and functional[19] differences.

As already shown in other animal species, during embryonic development a

progressive transition occurs in the relative abundance of different isoenzymes when these are viewed after electrophoretic separation.[11] In many mammalian species in early embryonic life the greatest concentration of LDH enzyme activity is localized in the negatively migrating fractions of LDH_4 and LDH_5 (M type) with progressive shifts of isoenzyme concentration in the various tissues as they differentiate and mature. Only one brief mention has been made in the literature to LDH isoenzyme studies in the human fetus.[20] Our studies in one newborn infant have confirmed the finding that in the human fetus the greatest concentration of isoenzymes is localized in the positive more rapidly migrating fractions of the H type (Fig. 4). This implies that in man the H type of subunits predominate early and that only later in development does the M type make its appearance in sizable proportion. Further studies on human fetuses of various ages must be done before these conclusions can be fully verified.

The specific physiological roles of the two separate types of LDH are not yet very clear. Several authors[11, 12] recently presented evidence which suggested different functions for the two types of LDH. The M type (i.e., LDH_5) has been found to be less sensitive to changes in pyruvate concentration than is the H type (i.e., LDH_1). Hence the M type, which is tolerant of high pyruvate concentration, is found in the tissues where glycolysis is the primary source of energy, e.g., in most of the skeletal muscles. Conversely, H type is present in the tissues which carry on mostly aerobic metabolism such as in the heart. Very recently, evidence has been presented in support of the hypothesis that these two different types of LDH are each controlled by a separate gene.[21]

The reasons for the diverse LDH isoenzyme patterns in different muscles in the adult are not fully understood. The predominance of LDH_5 in the thigh, psoas, deltoid, and pectoralis muscles may be due to the sudden participation of these muscles in maximum contraction and hence to their greater complement of white fibers. Since most of the muscles possess a mixture of all five types of LDH, the inherent heterogeneity, and yet specificity, of the individual muscles of the body is thus emphasized.

Summary.—The LDH isoenzyme patterns of normal human skeletal muscles and visceral organs have been found to vary greatly, ranging from predominant M type to H type. The human fetal type of LDH has been shown to be of the electrophoretically rapid migrating H type. During maturation and development after birth there occurs a shift toward the M type in most muscles like quadriceps, psoas, deltoid, and pectoralis, and in organs like the liver. MDH does not show any very characteristic pattern in different muscles and organs. In future studies on LDH isoenzymes in skeletal muscle it will be necessary to recognize both the specificity and the heterogeneity of that tissue.

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STUDIES ON THE PROTEIN COMPONENTS OF CILIA FROM TETRAHYMENA PYRIFORMIS

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Numerous studies with the electron microscope have established that all cilia and protozoan flagella possess essentially the same fine structure.[1, 2] However, interpretation of the function of this structure has been hindered by the fact that little is known about the chemistry of the constituents. ATP can induce motility in glycerinated cilia and flagella,[3, 4] and the isolated organelles possess ATPase activity,[4, 5] so that the energy for movement presumably comes from dephosphorylation of ATP. Work on the nature of ciliary proteins has been handicapped by their apparent insolubility at neutral pH.[5, 6] The experiments reported in this paper demonstrate that the apparent insolubility reported by previous workers is due to the membrane around the cilium, and that after removal of the membrane the remaining protein is soluble in salt solutions at neutral pH. Differential extraction and reconstitution of the ciliary structure, combined with electron microscopy, have made it possible to locate the probable site of ATPase activity. A partial fractionation of the protein components has been accomplished.

Materials and Methods.—Tetrahymena pyriformis, strain W, was grown at room temperature in a medium containing 1% peptone, 0.1% yeast extract, and phosphate buffer pH 6.5. The cilia were isolated by a procedure slightly modified from that of Watson and Hopkins,[6] and were stored in a small volume of tris-Mg solution. (Tris denotes tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate. Other abbreviations are those accepted as standard by the *Journal of Biological Chemistry*.)

Except where otherwise stated, all operations were carried out at 0–4°C. Two solutions which were used repeatedly will for convenience be designated as *tris-Mg solution* (2.5 mM $MgSO_4$, 0.03 *M* tris-HCl, pH 8.3 at 0°C), and *tris-EDTA solution* (0.1 mM EDTA, 1 mM tris-HCl, pH 8.3 at 0°C). Routine assays for ATPase activity were carried out by incubating at 20°C in a medium containing 1 mM ATP, 1.2 mM $MgSO_4$, and 0.03 *M* tris-HCl, pH 7.8. Subsequent assay for inorganic phosphate was by the method of Fiske and Subba-Row.[7] Protein assays were

performed by the method of Lowry *et al.*,[8] using a calibration curve prepared with a standard solution of bovine serum mercaptalbumin.[9] Undissolved material was normally removed from suspension by centrifuging at 35,000 *g* for 10 min; substances remaining in the supernatant are, for present purposes, defined as being in solution. Centrifuge pellets to be examined by electron microscopy were fixed with osmium tetroxide, embedded, and sectioned by the usual procedure.[1] Digitonin was obtained from Nutritional Biochemicals Corporation.

Results.—In order to present an intelligible account of the new observations, it is necessary to begin with a brief description of the characteristic fine structure of cilia and flagella; more detailed descriptions have been given elsewhere.[1, 2] The cilia of *Tetrahymena*, like other typical cilia, are approximately cylindrical and composed of a complex bundle of fibers which is embedded in a matrix and enclosed by a membrane (Fig. 1). The principal feature is the backbone of nine double outer fibers and two central fibers that runs continuously along the length of the cilium. Subfiber *A* of each outer doublet bears a double row of projections, called "arms," that extend toward subfiber *B* of the adjacent doublet. Between the outer and central fibers is another set of nine secondary fibers, smaller and less regular than the others. The three groups of fibers are held together at frequent intervals along the cilium by means of radially oriented links.

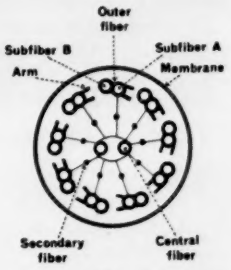


FIG. 1.—Diagram showing the structures visible in a cross section of a cilium. (Modified from ref. 1.)

Examination by light and electron microscopy showed that the preparations of isolated cilia contained no appreciable contamination with cell bodies or cytoplasmic debris. The isolation procedure caused little structural distortion of the cilia (Fig. 2), the only apparent alteration being that the membrane was more closely applied to the bundle of fibers than normal. The cilia could be kept in tris-Mg solution for a few hours without damage, but storage overnight resulted in many of the cilia losing their normal cylindrical shape and rounding up into spheres with the axial fiber bundle coiled around inside the membrane.

Preliminary experiments showed that very little protein was extracted from the

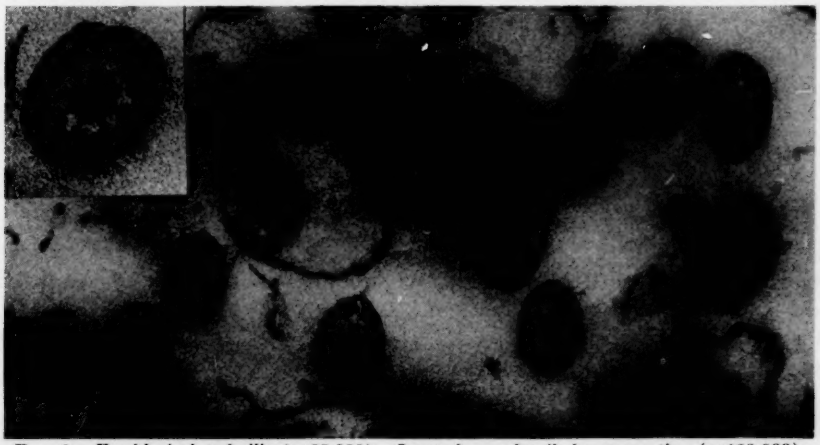


FIG. 2.—Freshly isolated cilia (×65,000). Inset shows detailed cross section (×100,000).

intact cilia by salt solutions at neutral pH. However, certain observations suggested that this apparent insolubility was due to the membrane enclosing the cilium rather than to inherent insolubility of the proteins themselves. Two methods have been used to break down the membrane, as a first step in obtaining the proteins in solution. The method to be described in this paper involves the selective solubilization of the membrane with digitonin, a surface-active glycoside. A second method, which involves disrupting the membrane by dialyzing the cilia against a low concentration of chelating agent, gave comparable results.

The results of extracting freshly isolated cilia twice with a 0.5 per cent solution of digitonin in tris-Mg solution are shown in Table 1. Since the second extraction

TABLE 1

EXTRACTION OF CILIA WITH DIGITONIN

	Total protein (mg)	Total ATPase activity (μmoles P/hr)	Protein in supernatant (mg)	ATPase activity in supernatant (μmoles P/hr)
Freshly isolated cilia in tris-Mg soln. Centrifuged.	4.8	15	0.6 (12%)	—
Pellet resuspended in 0.5% digitonin in tris-Mg soln. Centrifuged.	—	—	1.4 (30%)	2
Pellet resuspended in 0.5% digitonin in tris-Mg soln. Centrifuged.	—	—	0.3 (6%)	—
Pellet resuspended in tris-Mg soln.	2.4 (50%)	14	—	—

Figures in parenthesis represent percentages of initial protein.

with digitonin brings much less protein into solution than the first, it is reasonable to regard the ciliary protein as divisible into two fairly well-defined fractions, one soluble and one not soluble in digitonin under these conditions. The insoluble residue remaining after the second extraction will be called *digitonin-extracted cilia*, and the protein composing it will be called *structural protein*. The structural protein, which contains almost all the fibrillar ATPase activity, constitutes about 50 per cent of the total ciliary protein. When the pellet of digitonin-extracted cilia is examined by electron microscopy, the principal change apparent is that the membrane has been completely removed (Fig. 3). In most other respects the structure of the cilium appears to be intact and unaffected by the extraction. All the nonmembranous components shown in Figure 1 are still present, although slight indications of breakdown, such as a missing central fiber or a damaged outer fiber, can be seen in some cilia.

The structural protein of the cilium is almost wholly soluble in moderately concentrated solutions of salt. For example, a suspension of digitonin-extracted cilia in 0.6*M* KCl becomes gradually less turbid over a period of several hours as the protein goes into solution; after 18 hr 90–95 per cent of the protein and ATPase activity are in solution (Table 2), and centrifugation yields only a very small pellet composed mostly of residual lipid. The solution of structural protein has a typical protein absorption spectrum with a maximum near 276 mμ, and an absorbancy ratio 280/260 mμ of 1.3. Suspension in 0.6 *M* KI likewise brings almost all the protein into solution, but in this case the action appears instantaneous.

A useful fractionation of the structural protein is obtained by treating with a chelating agent at very low ionic strength. Dialysis of the digitonin-extracted cilia against tris-EDTA solution brings almost all the ATPase activity into solu-

FIG. 3.—Digitonin-extracted cilia (×65,000). Inset shows detailed cross section (×100,000).

FIG. 4.—The insoluble residue after dialyzing digitonin-extracted cilia against tris-EDTA solution (×65,000). Inset shows detailed cross section (×100,000).

TABLE 2

SOLUBILITY OF DIGITONIN-EXTRACTED CILIA AT pH 8.3

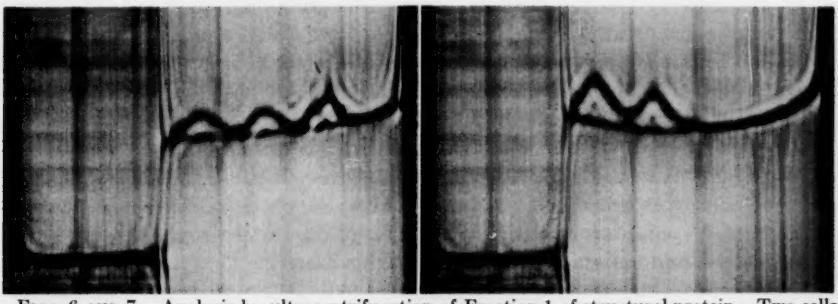
Extracting solution	Time of extraction	Per cent protein in solution	Per cent activity in solution	Specific activity of solution, μmoles P/(mg protein × hour)
0.6 *M* KCl, 30 mM tris-HCl, 2 mM $MgSO_4$	18 hr	95	90	5.3
1 mM tris-HCl, 0.1 mM EDTA	Dialyzed 18 hr	31	90	20
1 mM tris-HCl, 1 mM $MgSO_4$	18 hr	7	4	—
10 mM KCl, 30 mM tris-HCl, 1 mM EDTA	Dialyzed 18 hr	6	2	—
3 mM ATP, 30 mM tris-HCl, 2.5 mM $MgSO_4$	5 min	5	—	—

tion, but only about 30 per cent of the protein (Table 2). The soluble fraction obtained by this dialysis will be denoted *Fraction 1* of the structural protein; the residue, insoluble in tris-EDTA solution, will be called *Fraction 2*. Examination of this insoluble residue in the electron microscope shows it consists of the outer fibers alone (Fig. 4); the other structural components, including the arms on the outer fibers, have been almost completely removed. It is remarkable that the arrangement of outer fibers found in an intact cilium still largely persists. Thus, many of the outer fibers occur in sets of nine, grouped around the periphery of a cylinder 0.2 μ in over-all diameter; even details of their arrangement such as their slight inward twist are maintained. It seems that the outer fibers are held together in these cylindrical groups by means of a thin filament running around the inside of the cylinder from one *A* subfiber to the next. Not all the cylinders in a preparation are intact; frequently the wall is broken open at one place, and sometimes smaller groups of 1–8 outer fibers occur. There is no reason to doubt that the connections between *A* subfibers are present also in native cilia, although they are very difficult to see and have not been described previously. Since Fraction 2 of the structural protein contains little ATPase activity, it is evident that the enzyme is not located in the outer fibers but in one of the components removed by dialysis.

We have seen that the digitonin-extracted cilia are partially soluble at very low ionic strength and that they are completely soluble at moderately high ionic strength. Between these extremes, however, the structure is more stable. Thus, 1 mM $MgSO_4$ is sufficient to maintain it intact (Table 2). Divalent ions are not essential, however, for the structure is also stabilized by 10 mM KCl in the presence of EDTA. Only a small amount of protein goes into solution when the digitonin-extracted cilia are treated with ATP.

It seemed of interest to see whether any reconstitution of the fine structure could be obtained by restoring magnesium to the dialyzed preparation. In one such experiment, a preparation of digitonin-extracted cilia was dialyzed against tris-EDTA solution and then divided into two. To one half, tris buffer and $MgSO_4$ were added to make the concentrations up to the standard tris-Mg solution; the other half of the preparation was left in tris-EDTA solution as a control. After 90 min, the two were centrifuged, the supernatants assayed for ATPase activity and for protein, and the pellets fixed for electron microscopy. In the preparation to which magnesium had been restored, 17 per cent of the total protein and 42 per cent of the total activity were in solution, while in the control 31 per cent of the protein and 88 per cent of the activity were in solution. These results show that about half of the protein and half of the activity which went into solution on dialysis have become re-bound to the insoluble residue. Electron micrographs of the pellets from this same experiment are shown in Figures 4 and 5. The principal structures to have returned in the reconstituted preparation are the arms on the outer fibers. These appear to have returned with a remarkable degree of precision to the same position that they had in the intact cilia, with one pair of arms located on subfiber *A* of each outer fiber (Fig. 5). It is not yet possible to say what percentage of the arms has returned on reconstitution, partly because their distribution along the length of the outer fibers is difficult to determine. The precision with which the arms return illustrates the morphogenetic potential inherent in the recombining units and is relevant to the question of how cilia are formed. In addition to the

FIG. 5.—"Reconstituted" cilia obtained by restoring magnesium to a preparation of digitonin-extracted cilia that had been dialyzed against tris-EDTA solution (×50,000); insets show portion of same micrograph (×100,000). (The same preparation of dialyzed cilia but without addition of magnesium is shown in the upper inset of Fig. 4.)



FIGS. 6 AND 7.—Analysis by ultracentrifugation of Fraction 1 of structural protein. Two cells were run, one containing sample, the other a solvent blank. Figure 6 shows Fraction 1 in tris-EDTA solution. Figure 7 shows the same preparation of Fraction 1 in tris-EDTA solution containing 0.1 *M* Na_2CO_3. Kel-F centerpiece; 59,780 rpm; 20°C; bar angle, 50°; time: 16 min after reaching full speed.

structures which return specifically to their original position, there is also some nonspecific adsorption. This appears as "fluffy" material coating the outer fibers.

The above evidence indicates that the presence or absence of ATPase activity in the ciliary residue is correlated with the presence or absence of the arms on the outer fibers. The correlation strongly suggests that the arms are the site of at least part of the fibrillar ATPase activity of the cilium (see Discussion).

Analysis by ultracentrifugation, and further purification of the ATPase: When Fraction 1 of the structural protein is examined by analytical centrifugation, three principal peaks are visible with approximate sedimentation coefficients ($s_{20,w}$) of 4, 13, and 25 S (Fig. 6), together with a minor peak at about 45 S. The 4 S and 13 S peaks are symmetrical, but the fastest (25 S) peak frequently has a trailing shoulder indicating the presence of some material sedimenting in the range 20–25 S. As the 25 S peak crosses the field, it appears to spread rather more slowly than the other two peaks, and it bears a characteristic hypersharp tuft on its crest; these properties suggest a degree of asymmetry in the 25 S particles. The relative area of the 13 S and 25 S peaks appears to vary somewhat in different preparations of Fraction 1. In the preparation shown in Figure 6, for example, the two have approximately the same area, but in some other preparations the 13 S peak was much smaller relative to the 25 S peak. The factors responsible for this variation are not yet known. Substantially the same sedimentation pattern is seen whether the Fraction 1 is run in tris-EDTA solution, in tris-EDTA solution containing 0.1 *M* KCl, or in tris-Mg solution. Addition of 3×10^{-3} *M* ATP to the sample in the centrifuge cell immediately before a run in tris-Mg solution had no effect on the pattern.

The variation in the relative size of the 13 S peak suggested that it might be a breakdown product of the 25 S peak. This hypothesis was confirmed by running Fraction 1 in alkali (0.1 *M* Na_2CO_3) instead of at the usual pH 7.8;[10] then only two peaks sedimenting at about 3.5 S and 11 S are seen (Fig. 7). These two peaks probably correspond to the 4 S and 13 S peaks of the usual pattern, the slight decrease in sedimentation constant being due to the difference in pH. Comparison of area under the peaks shows that the total area is about the same at both pH's, with the 25 S peak in Figure 6 being about equally divided between the 4 S and 13 S peaks in Figure 7. As the same preparation of Fraction 1 was used, this result clearly shows that treatment with alkali breaks down the 25 S particles to a mixture of 4 S and 13 S. However, the factors determining the presence of both 13 S and 25 S particles in all the normal preparations are still unknown.

A solution of Fraction 2 in 0.6 *M* KCl showed a single peak sedimenting at about 4 S, together with a large amount of heterogeneous material sedimenting in the range 4–50 S. A solution of whole digitonin-extracted cilia in 0.6 *M* KCl showed the same pattern as would be obtained by adding the patterns of Fractions 1 and 2 run separately; addition of 3×10^{-3} *M* ATP to the sample in the centrifuge cell immediately before running had no effect on this pattern.

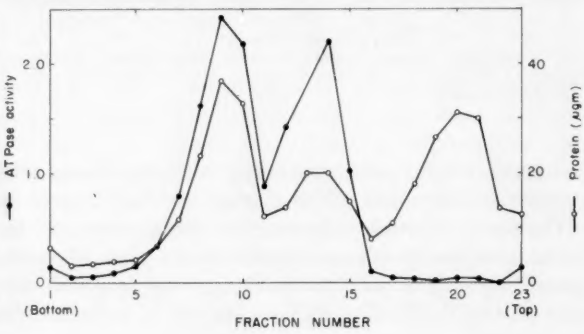


FIG. 8.—Separation of the components of Fraction 1 by centrifugation through a sucrose density gradient. 0.1 ml of Fraction 1 in tris-EDTA solution layered on top of a 5–30% sucrose gradient, and centrifuged 7 hr at 38,000 rpm in SW 39 rotor of Spinco centrifuge. Contents of centrifuge tube collected in samples of three drops each. ATPase activity in μmole P/hour. No activity measurement sample 13.

The three principal components of Fraction 1 have been separated by zonal centrifugation through a sucrose density gradient.[11] Analysis for protein showed that the 4, 13, and 25 S components were well resolved (Fig. 8). All three components had typical protein absorption spectra with maxima near 280 mμ and absorbancy ratios 280/260 mμ of 1.3–1.4. The ATPase activity was about equally divided between the 13 S and 25 S components; the 4 S was completely inactive (Fig. 8). The specific activities of the 13 S and 25 S components were 77 and 40 μmole P/(mg protein × hour), respectively (average of four experiments). Taken with the other evidence above, the higher specific activity of the 13 S component indicates that it is the enzymatically active portion liberated on breakdown of the 25 S particles. The 13 S component is the most highly purified form of the fibrillar ATPase prepared so far. It has a specific activity about 15 times higher than that of whole cilia, and represents about 8 per cent[12] of the total ciliary protein.

Discussion.—Now that the ciliary proteins can be obtained in solution under mild conditions they become amenable to study by standard physical-chemical methods. However, it should not be assumed that one is necessarily dealing with true monomeric molecules, for the very fact that the proteins occur aggregated together in the structure of the cilium suggests that similar, although smaller, aggregates and complexes may also occur in solution. It is not yet known how many distinct proteins are present in the structural protein fraction. The experimental data so far require at least two (one with ATPase activity and sedimenting at about 13 S, the other with no ATPase activity and sedimenting at about 4 S), but there may well be more. The 25 S particles found in solutions of structural protein probably represent a naturally occurring subunit of the cilium, one which is partially broken down in the normal course of preparation, and which is wholly broken down to a mixture of 4 S and 13 S particles by 0.1 *M* Na_2CO_3.

When studying the chemistry of cilia, it is customary to look for analogies with the chemistry of muscle. The properties of Fraction 2 of the structural protein have some likeness to those of actin, while the ATPase of cilia appears to resemble the ATPase of the mitotic apparatus[13] more than it does that of myosin. However, the value of comparisons based on such fragmentary evidence is open to question.

Current hypotheses usually assume that the bending movements of cilia are the result of the localized shortening of longitudinal contractile elements,[1, 14] and the experiments reported here are consistent with a mechanism of this type. Since the immediate source of energy for motility is provided by dephosphorylation of ATP, it seems probable that the mechanical force responsible for motility must be initially generated within monomolecular distance of the site of ATPase activity. This suggests that the longitudinal elements which contract are the outer fibers and that the contractions result from the interaction of the arms with two adjacent outer fibers. The forces resulting from a contracted region of one outer fiber would thus be transmitted automatically to the adjacent outer fibers on each side, and this would account for the spread of contractions around the cilium which is necessary to account for the observed bending movements.

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